Dense Bodies of a gH/gL/UL128/UL130/UL131 Pentamer-Repaired Towne Strain of Human Cytomegalovirus Induce an Enhanced Neutralizing Antibody Response

Caroline Lehmann,^{a*} Jessica Julia Falk,^b Nicole Büscher,^a Inessa Penner,^a Christine Zimmermann,^a Patricia Gogesch,^a Christian Sinzger,^b Bodo Plachter^a

^aInstitute for Virology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

^bInstitute for Virology, Ulm University Medical Center, Ulm, Germany

ABSTRACT The development of a vaccine against human cytomegalovirus infection (HCMV) is a high-priority medical goal. The viral pentameric protein complex consisting of glycoprotein H (gH)/gL/UL128-131A (PC) is considered to be an important vaccine component. Its relevance to the induction of a protective antibody response is, however, still a matter of debate. We addressed this issue by using subviral dense bodies (DBs) of HCMV. DBs are exceptionally immunogenic. Laboratory HCMV strain DBs harbor important neutralizing antibody targets, like the glycoproteins B, H, L, M, and N, but they are devoid of the PC. To be able to directly compare the impact of the PC on the levels of neutralizing antibody (NT-abs) responses, a PC-positive variant of the HCMV laboratory strain Towne was established by bacterial artificial chromosome (BAC) mutagenesis (Towne-UL130rep). This strain synthesized PC-positive DBs upon infection of fibroblasts. These DBs were used in side-by-side immunizations with PC-negative Towne DBs. Mouse and rabbit sera were tested to address the impact of the PC on DB immunogenicity. The neutralizing antibody response to PC-positive DBs was superior to that of PC-negative DBs, as tested on fibroblasts, epithelial cells, and endothelial cells and for both animal species used. The experiments revealed the potential of the PC to enhance the antibody response against HCMV. Of particular interest was the finding that PC-positive DBs induced an antibody response that blocked the infection of fibroblasts by a PC-positive viral strain more efficiently than sera following immunizations with PC-negative particles.

IMPORTANCE Infections with the human cytomegalovirus (HCMV) may cause severe and even life-threatening disease manifestations in newborns and immunosuppressed individuals. Several strategies for the development of a vaccine against this virus are currently pursued. A critical question in this respect refers to the antigenic composition of a successful vaccine. Using a subviral particle vaccine candidate, we show here that one protein complex of HCMV, termed the pentameric complex (PC), enhances the neutralizing antibody response against viral infection of different cell types. We further show for the first time that this not only relates to the infection of epithelial or endothelial cells; the presence of the PC in the particles also enhanced the neutralizing antibody response against the infection of fibroblasts by HCMV. Together, these findings argue in favor of including the PC in strategies for HCMV vaccine development.

KEYWORDS cytomegalovirus, dense bodies, gH/gL/UL128-131, neutralizing antibodies, pentamer, vaccine

Congenital human cytomegalovirus infection (cCMV) is a major cause of childhood disease, often leading to permanent neurologic sequelae (1). As a consequence, current research efforts focus on the development of prophylactic vaccines against

Citation Lehmann C, Falk JJ, Büscher N, Penner I, Zimmermann C, Gogesch P, Sinzger C, Plachter B. 2019. Dense bodies of a gH/gL/UL128/UL130/UL131 pentamer-repaired Towne strain of human cytomegalovirus induce an enhanced neutralizing antibody response. *J Virol* 93:e00931-19. <https://doi.org/10.1128/JVI.00931-19>.

Editor Rozanne M. Sandri-Goldin, University of California, Irvine

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Bodo Plachter, plachter@uni-mainz.de.

* Present address: Caroline Lehmann, EMBL Ventures GmbH, Heidelberg, Germany.

C.L. and J.J.F. are co-first authors. C.S. and B.P. are co-senior authors.

Received 5 June 2019

Accepted 5 June 2019

Accepted manuscript posted online 12 June 2019

Published 13 August 2019

chCMV. Such a chCMV vaccine has been identified as a top-priority medical goal (2, 3). Various vaccine candidates have been developed and are currently tested in preclinical or clinical studies (reviewed in references 4–7). Although some of these candidates have shown a moderate protective effect against primary infection, there are still open questions regarding the goals and the most appropriate formulation for a successful HCMV vaccine (8).

One controversially discussed issue is the protective potential of virus-neutralizing antibodies (NT-abs) directed against different viral envelope proteins. The glycoproteins B (gB) and H (gH) had been identified as major NT-abs targets (9–11). Both proteins, particularly gB, are thus considered to be important for vaccine development (7, 12, 13). The limited protection observed with a gB subunit vaccine (12, 14) suggests the need for additional antigens for successful vaccination. The protein complex consisting of the glycoproteins gH, gL, pUL128, pUL130, and pUL131 (PC) has been identified as one such promising antigenic component (15, 16). The PC appears to be a major target of the NT-abs response in humans, as a large fraction of the NT-abs activity against HCMV in reconvalescent sera is directed against this complex (17). The rationale behind this is the finding that the PC is important for HCMV infection of different cell types, such as endothelial cells, epithelial cells, or dendritic cells (15, 16, 18, 19). In contrast, infection of fibroblast cell cultures proceeds independently of the presence of the PC but requires gB as well as a trimeric complex consisting of gH/gL/gO (PC-independent infection).

One focus of our interest is directed toward the understanding of the immune response against HCMV infection and, related to that, the development of an HCMV vaccine candidate based on subviral dense bodies (DBs) of HCMV. These particles are synthesized in and released from human fibroblast cell cultures (20, 21). They are devoid of viral capsids and viral DNA (22). DBs are composed of an internal electron-dense structure mainly consisting of viral tegument proteins and a lipid envelope with the viral surface protein complexes inserted in a fusion-competent conformation (21, 23–26). The DBs can be modified for the upload of additional proteins or antigenic peptides (27, 28). Despite being replication incompetent, DBs induce considerable CD4⁺ and CD8⁺ T lymphocyte responses when evaluated in preclinical animal models (25, 28, 29). This is likely related to the maturation and activation of dendritic cells (DCs) following incubation of these cells with DBs (30). DB application also induces high levels of NT-abs against HCMV infection (25, 28, 29, 31). Since viral envelope proteins are inserted in their functional conformation into the envelopes of DBs, these particles likely induce humoral responses comparable to those induced by viral infection.

All previous experiments using purified DBs as immunogens have been conducted with particles from laboratory HCMV strains which are devoid of the PC (25, 27–29, 31–34). Here, we attempted to analyze DBs containing the PC (PC-positive) alongside PC-negative DBs in order to address the contribution of PC-specific antibodies to DB-induced neutralizing antibody responses. For this, a DB-producing HCMV strain based on the laboratory strain Towne was established. This strain stably expressed the PC also after passaging in human foreskin fibroblast (HFF) cell cultures. PC-positive DBs from this strain, compared with PC-negative DBs, indeed proved to be superior in their potential to induce NT-abs responses against HCMV infection of ARPE-19 epithelial cells and HEC-LTT endothelial cells. Surprisingly, the sera from animals that had been immunized with PC-positive DBs also showed a higher neutralization activity against infection of fibroblasts with a PC-positive HCMV strain, indicating that infection of fibroblasts also is, to some extent, affected by the PC.

(Part of this research was conducted by C. Lehmann [née Sauer] in fulfillment of the requirements for a doctoral degree from the Johannes Gutenberg University, Mainz, Germany, 2013 [68].)

RESULTS

Antibodies induced by laboratory strain DB immunization neutralize the PC-positive strain TB40/E with comparable efficiencies in HFF and ARPE-19 epithelial cell cultures. HCMV envelope proteins are expressed on the surface of laboratory strain

FIG 1 Microneutralization analysis of the neutralizing antibody response of rabbits following laboratory strain DB (DB-HB5) immunization. The assay was performed by counting the reduction of IE1-positive cells following antibody incubation. (A) Schematic representation of the immunization protocol. (B to D) NT₅₀ (dashed line) of individual sera against HB5 on HFF (B), TB40/E on HFF (C), and TB40/E on ARPE-19 (D) cells. Depicted are the results from four technical replicates. The NT₅₀ was defined as the first serum dilution in each case that reduced the number of IE1-positive cells by more than 50%. The results shown are representative for the reactivity of the sera from one out of two immunized animals.

DBs in a conformation suitable to mediate entry of the particles into human fibroblasts by membrane fusion (25, 26). The laboratory strain DBs, however, display little efficiency in penetrating epithelial cells (C. Lehmann, unpublished data), likely related to the absence of the PC on their surface, resembling observations on HCMV laboratory strains that fail to infect epithelial cells (35). In a first set of experiments, we investigated the level of neutralization against a PC-positive viral strain by sera from animals that had been immunized with PC-negative DBs. New Zealand White rabbits were immunized with laboratory strain DBs (DB-HB5) according to the schedule displayed in Fig. 1A. Sera were collected at the indicated time points and tested in microneutralization assays. As expected, the sera neutralized the PC-negative homologous strain HB5 on fibroblasts already at a low antibody concentration (Fig. 1B). The sera also neutralized the PC-positive strain TB40/E-BAC_{KL7} (here TB40/E) on HFFs (Fig. 1C), although a higher antibody concentration was necessary. Similar 50% neutralizing titers (NT₅₀) were detected when ARPE-19 cells were used as targets, indicating that the sera were equally effective blocking TB40/E infection in HFF and epithelial cells (Fig. 1D). Taken together, these results showed that rabbit sera induced by HCMV laboratory strain DBs contain neutralizing antibodies that impair viral infection of TB40/E on both HFF and ARPE-19 cells. They also indicated that the level of neutralization of TB40/E was comparable for the two cell types, despite the lack of the PC in the material used for immunization.

Generation of a PC-positive variant of the HCMV Towne strain. The initial experiments and the results from others (29) indicated that the fully assembled envelope protein complexes of laboratory strain DBs lacking the PC already induced a distinct neutralizing antibody response against infection by a PC-positive strain. The PC had, however, been identified as a dominant target of the NT-abs response against HCMV. We thus designed experiments to analyze the impact of antibodies against the PC using side-by-side immunization with PC-positive and PC-negative DBs. To be able to directly compare the results, we decided to use a pair of viruses for DB production that only differed in PC expression. As a basis for this, we selected the Towne strain, which is a high-level DB producer. The Towne strain carries a double adenine insertion in the open reading frame (ORF) of the UL130 gene, leading to a frameshift (16, 36). The parental Towne strain is thus PC negative. To generate a Towne variant that expresses the PC, the mutated UL130 open reading frame in the BACmid pTowne (37) was

replaced by its functional homolog from the TB40/E strain, using the *galk* negative-positive selection procedure (38). The resulting BACmid was denominated pTowne-UL130rep (Fig. 2A). We chose TB40/E UL130, as the expression of the PC by TB40/E appears to be conserved upon serial passaging in fibroblast cell cultures (C. Sinzger, unpublished data) (39, 40). Following reconstitution on HFF, virions and DBs of the novel strain Towne-UL130rep were purified by gradient ultracentrifugation. These particles were subjected to immunoblot analysis and probed with antibodies against pUL130, pUL128, and glycoprotein B (loading control, Fig. 2B). Particles from the laboratory strains HB5, the parental strain Towne, and from TB40/E were carried along as a control. pUL130 and pUL128 were both detectable in virions and DBs of Towne-UL130rep. According to current knowledge, both proteins are inserted into the envelope of extracellular viral particles only in complex with gH/gL and UL131. This suggested that the PC was successfully restored in Towne-UL130rep. To formally prove the functionality of the complex, ARPE-19 cells were infected with Towne-UL130rep. Infection efficiency was compared to parallel cultures infected with the parental Towne strain or with TB40/E and was measured by counting immediate early protein 1 (IE1)-positive cells (Fig. 2C). The median number of IE1-positive cells in the TB40/E-infected cultures was set to 100%. The infection rate of Towne-UL130rep was comparable to that of TB40/E. The parental Towne strain showed less than 20% IE1-positive cells, compared to the rate of positive cells, following TB40/E infection in that culture. Since the PC is considered to be important for the infection of epithelial cells, these results provided further evidence for successful reconstitution of the PC in Towne-UL130rep.

To further address this issue, ARPE-19 cells were tested for their capacity to support Towne-UL130rep DNA replication. Cells were infected with Towne or Towne-UL130rep, using identical input genome copy numbers. HFFs were infected in parallel (Fig. 2D). Genome replication was assessed by quantitative PCR analysis. Towne-UL130rep genome replication was detectable in epithelial cells. No replication of the parental strain Towne was seen. Replication was detectable to high levels in HFF, with little difference between Towne and Towne-UL130rep. To address whether virus was released from Towne-UL130rep-infected epithelial cells and HFFs, culture supernatants were probed for viral genomes, again using quantitative PCR analysis (Fig. 2E). Supernatants from Towne-UL130rep-infected epithelial cells contained increasing amounts of viral genomes over time, indicating that infectious virus was being released. In contrast, a decrease in the genome copy numbers was seen in Towne-infected ARPE-19 cultures. HFFs infected with either strain released equal genome copy numbers. Taken together, these data showed that the Towne strain had been functionally repaired in its capacity to replicate in epithelial cells and to release progeny from these cultures.

To analyze if the phenotype of Towne-UL130rep resembled that of other PC-positive HCMV strains, we investigated the cytopathogenic changes that were induced by that virus on HFFs, using immunofluorescence analyses. Restoration of the PC in the laboratory strain AD169 is associated with the appearance of multinucleated giant cells (35). It is also a characteristic of the PC-positive strain TB40-E (C. Sinzger, unpublished data). To verify that Towne-UL130rep shared that phenotype, HFFs were infected for 8 days. Cells were then tested for the induction of cell-cell fusion by indirect immunofluorescence staining, using labeling with a pp65-specific monoclonal antibody for detection (Fig. 3). Towne-UL130rep-infected HFFs indeed formed large multinucleated cells which were absent in Towne-infected cells. Substantial cell-cell fusion was also seen in cultures that were infected with TB40/E for a control. These results confirmed that the repair of UL130 in the HCMV strain Towne created a virus that was phenotypically indistinguishable from other PC-positive viruses.

Sera from PC-positive DB-immunized animals display higher neutralization capacities against fibroblast infection. Mass spectrometry of purified DBs of strains Towne and Towne-UL130rep confirmed that both particles differed only with respect to the presence of the UL128-131 (not shown). DB preparations from the two viruses were

FIG 2 Establishment of a UL128-131-gH-gL competent DB producer strain of HCMV. (A) Schematic representation of the cloning strategy. The genomic region encoding the nonfunctional UL130 gene of HCMV strain Towne (carrying a double adenine insertion) was replaced in Towne BAC by a galactokinase gene (*galK*) expression cassette. After positive selection in *E. coli*, the intermediate bacmid pTowne-UL130galK was then used for insertion of a functional UL130, PCR amplified from the HCMV TB40/E genome. The primers used for amplification procedures are indicated. The resulting BACmid pTowne-UL130rep was then used for virus reconstitution in HFF. The resulting virus was labeled as Towne-UL130rep. (B) Immunoblot analysis of glycerol-tartrate gradient-purified extracellular virions (V) and DBs from Towne-UL130rep. The respective particles of strains HB5, Towne BAC (Towne), and TB40/E were carried along for control. Filters were incubated with antibodies against UL130, UL128, and glycoprotein B (gB). Three micrograms of the respective particle fractions was applied to each slot. The molecular masses of the size standard are indicated. (C) Phenotypic analysis of UL128-131-gH-gL expression on Towne-UL130rep virions. ARPE-19-cells were infected with an MOI of 0.2 of Towne-UL130rep. The cells were also infected with the same MOIs of strains Towne BAC and TB40/E for control reasons. The cells were stained with an IE1-specific antibody at 24 h after infection. Five randomly selected sections were microscopically inspected and counted for IE1-positive cells. The mean calculated for TB40/E-BAC was

(Continued on next page)

FIG 3 Indirect immunofluorescence analysis of the reestablishment of cell fusion activity of the Towne strain by UL130 repair. HFF cells were infected with the indicated strains for 8 days. Cells were either stained with a pp65-specific monoclonal antibody (top row) or with 4',6-diamidino-2-phenylindole (DAPI) (bottom row).

thus perfectly suitable to investigate the difference in antibody responses mediated by the presence of a functionally active PC.

In a first set of experiments, differences in the induction of neutralizing antibodies following immunization with PC-positive (PC-pos) versus PC-negative (PC-neg) DBs were addressed on fibroblasts. In a first round of immunizations, rabbits were immunized with 25 μ g DB-Towne-UL130rep or DB-Towne according to the schedule shown in Fig. 4A. The sera from these animals, collected at days 66 and 86, were analyzed for their virus neutralization capacity using TB40/E for infection and immunofluorescence staining of viral IE antigen as readout. Sera from animals that received DB-Towne-UL130rep neutralized TB40/E significantly more efficiently on fibroblasts than did sera following application of DB-Towne (Fig. 4B and C). To corroborate these findings, another immunization experiment was performed in rabbits according to a shorter schedule using another DB batch (Fig. 4D). For analysis of these sera, a neutralization (NT) assay, based on the detection of *Gaussia* luciferase expressed by the reporter virus TB40-BAC4-IE-GLuc, was applied (41). Again, DB-Towne-UL130rep induced a significantly more efficient neutralizing response, especially at day 28 after the first immunization, additionally reflecting an enhanced boosting capacity. Taken together, these results suggest that PC-positive DBs are superior in the induction of neutralizing antibodies against fibroblast infection by a strain that contains the complete set of envelope proteins (TB40/E).

Sera from PC-positive DB-immunized animals display higher neutralization capacities against epithelial and endothelial cell infection. Both epithelial cells and endothelial cells are key targets of HCMV infection *in vivo* (42). An important issue thus was to evaluate whether the application of PC-pos DBs increased the neutralization capacity against infection of these cells. In a first experiment, mice were immunized twice intraperitoneally in a 2-week interval with DB-Towne or DB-Towne-UL130rep. Samples were collected at the end of week 4. The serum samples of 5 mice in each group were pooled and analyzed in microneutralization assays on ARPE-19 cells. Sera

FIG 2 Legend (Continued)

taken as 100%. (D) Genome replication of Towne-UL130rep in HFF and ARPE-19 cells. A total of 5×10^5 cells were infected with an MOI of 0.1. The cells were collected at the indicated times of infection. DNA was isolated and subjected to quantitative PCR analysis. The replication of Towne-Bac was measured alongside for control. The means of three independent technical replicates are shown. (E) Viral DNA release from Towne-UL130rep-infected HFF and ARPE-19 cells. Cells were infected as described in panel D. Culture supernatants were collected at the indicated times of infection and frozen for further analysis by quantitative PCR. Genome release from Towne BAC-infected cells was measured in parallel for a control. The means of three independent technical replicates are shown.

FIG 4 Difference in neutralization capacities on HFF of sera from rabbits immunized with PC-pos (Towne-UL130rep) or PC-neg (Towne) DBs. Rabbits were immunized four times with 25 μ g of DBs according to the schedules displayed in panel A or D. d, days. The individual bleeds were tested in neutralization assays on HFF using the viral strain TB40/E. (B and E) Dose response curves show mean values from three (B) or four (E) independent assays. (C and F) For bar graphs, curves from individual experiments were analyzed by nonlinear regression, and bars represent the mean values of the serum dilutions that reduced infection by 50% (NT₅₀). Error bars represent standard errors of the mean values. **, $P \leq 0.01$; *, $P \leq 0.05$.

FIG 5 Microneutralization assay of the antibody response of mice following immunization with PC-pos or PC-neg DBs. Mice were immunized twice at a 2-week interval with different DBs and were bled at 4 weeks. Sera from 5 mice in each group were pooled, heat inactivated at 56°C for 30 min, and used for neutralization assays. (A) NT₅₀ values of the neutralization of strain Towne-UL130rep by the sera from mice immunized with DB-Towne-UL130rep. (B) NT₅₀ values of the neutralization of strain Towne-UL130rep by the sera from mice, immunized with DB-Towne. The mean values of 4 technical replicates are indicated by bars. NT₅₀ values are indicated by dashed lines.

from mice that had been immunized with DB-Towne-UL130rep showed higher neutralization capacity on ARPE-19 cells than did the sera from animals that had been immunized with DB-Towne (Fig. 5). The results showed indeed that PC-positive DBs induced a more potent NT-abs response in mice which is protective against the infection of epithelial cells with a PC-pos HCMV strain.

To confirm these results in another animal species and on different cells, the sera from rabbits that had been used for the analysis on HFFs (see Fig. 4) were also used for neutralization assays on HEC-LTT endothelial cells (43, 44). The sera obtained after a four-dose application schedule (Fig. 6A) of DB-Towne-UL130rep (Towne-UL130rep) and sampling at day 66 or 86 showed enhanced neutralization capacity against strain TB40/E infection on endothelial cells, compared to sera from animals immunized with DB-Towne (Fig. 6B and C, Towne). To confirm these results, the sera from the independent immunization experiment (see Fig. 4) were tested accordingly. Again, sera obtained at two different times after application of DB-Towne-UL130rep showed enhanced neutralization of strain TB40/E on endothelial cells compared to DB-Towne application. Taken together, the experiments demonstrate the potential for the PC to enhance and broaden DB immunogenicity against HCMV.

Generation of a seed virus strain for DB production purposes. The BAC vector, originally used for the cloning of Towne BAC, expresses the green fluorescent protein (GFP). This protein is fortuitously packaged into DBs of Towne BAC and of Towne-UL130rep (proteomic analysis, not shown). To avoid the potential risk of adverse effects of GFP following DB application to humans, we deleted the GFP gene from Towne-UL130rep in order to establish a seed virus strain for the production of DBs for clinical studies. The *galk* gene was used to replace the GFP-gene in Towne-UL130rep, again using BAC technologies (Fig. 7A). The *galk* gene is driven by a prokaryotic promoter and is thus not expressed in eukaryotic cells. The resulting viral strain was denominated Towne-UL130rep-ΔGFP. The expression of pUL130, representative of PC expression, on purified DBs and virions was tested by immunoblotting (Fig. 7B). Abrogation of GFP expression was tested both by direct immunofluorescence (not shown) and by immunoblotting using a GFP-specific antibody (Fig. 7B). The functionality of the PC was verified by infecting both HFF and ARPE-19 cells with Towne-UL130rep-ΔGFP and staining for IE1 expression, using indirect immunofluorescence analyses (Fig. 7C). Taken together, these results showed that the newly established seed virus strain Towne-UL130rep-ΔGFP lacked expression of the green fluorescent protein but retained PC expression. These data show that DBs of strain Towne-UL130rep-ΔGFP are suitable for the production of a DB vaccine candidate for clinical studies.

FIG 6 Difference in neutralization capacities on HEC-LTT of sera from rabbits immunized with PC-pos (Towne-UL130rep) or PC-neg (Towne) DBs. Rabbits were immunized four times with 25 μ g of DBs according to the schedules displayed in panel A or D. The individual bleeds were tested in neutralization assays on HEC-LTT using the viral strain TB40/E. (B and E) Dose response curves show values of one assay (B) or mean values of three independent assays (E). (C and F) For bar graphs, curves from individual experiments were analyzed by nonlinear regression, and bars represent mean values of the serum dilutions that reduced infection by 50% (NT₅₀). Error bars represent standard errors of the mean values. *, $P \leq 0.05$, ns not significant.

FIG 7 Generation of a GFP-negative version of Towne-UL130rep. (A) Schematic representation of the BAC-cloning strategy to establish Towne-UL130repΔGFP. (B) Immunoblot analyses of the packaging of gpUL130 and of the lack of GFP packaging, respectively, into Towne-UL130repΔGFP DBs. (C) Indirect immunofluorescence analysis of the infection of ARPE-19 by Towne-UL130repΔGFP. Towne-UL130rep served as positive control and Towne BAC as a negative control.

DISCUSSION

We and others have shown that DBs are highly immunogenic, bearing the exceptional capacity to induce both humoral and cellular immune responses without requiring an adjuvant (25, 28, 29, 31, 33, 34). DBs induce maturation and activation of immature dendritic cells in addition, likely explaining their impact on the priming of immune responses (30). It remained unclear, however, whether the presence of the PC in DBs would enhance their immunogenicity.

Most studies on DB-induced immune responses were conducted with PC-negative DBs. An unpurified mixture of PC-positive and PC-negative virions and DBs was used for immunization in one study (31). The results did not allow any conclusion regarding the impact of the PC in DBs with respect to their immunogenicity. The strategy used in this work enabled, for the first time, an evaluation of the value of the PC in a prospective DB vaccine. In immunization experiments in mice and in repeated immunizations in rabbits, sera obtained after PC-positive DB application had a higher neutralization capacity against infection of endothelial and epithelial cells, as well as fibroblasts, than did sera obtained after PC-negative DB application. These cells are considered to be major targets of HCMV infection *in vivo*. Protection against infection of these cells is thus a major issue in vaccine development.

PC-positive DBs show superior immunogenicity with respect to the induction NT-abs. The PC has been identified as a major target of the NT-abs response during natural HCMV infection (17). Dominant neutralizing epitopes have been described on the PC (45). A recombinant PC expressed in CHO cells induced antibodies in mice with higher neutralization capacities than with antibodies induced by gB immunization (19). A multiantigenic PC-incorporating modified vaccinia Ankara (MVA) vector vaccine candidate is able to induce neutralizing antibodies as well as mouse and human major histocompatibility complex (MHC)-restricted polyfunctional T cell responses (46). Detection of PC-specific antibodies in human serum may correlate with virus control *in vivo* and with decreased incidence of HCMV transmission in pregnancy (47, 48). Still, the issue of whether the PC is required for a successful vaccine formulation in general remains unsettled. Vaccines like gB/MF59 provided some levels of protection against infection, fostering efforts to solely use gB for further development (12, 14). In the light of the above-mentioned findings, most currently pursued approaches, however, favor the addition of the PC to an HCMV-targeting vaccine (4, 6, 46, 49–53). The data

obtained herein appear to support this notion. Although PC-negative DBs induced moderate NT-abs responses against the infection of epithelial and endothelial cells, the responses to PC-positive DBs were superior in both animal species tested. This suggests that the PC is required on DBs to induce a broad NT-abs response. The data clearly support efforts to evaluate the potential of PC-positive DBs in clinical studies in humans.

The PC enhances the NT-abs response against TB40/E infection of fibroblasts.

Besides epithelial and endothelial cells, fibroblasts have also been identified as target cells of HCMV *in vivo* (54). Infection of these cells is considered to be mediated by membrane fusion at the cell surface, although work described in a recent publication would argue in favor of macropinocytosis for this entry pathway (55). In any case, PC-negative laboratory strains and PC-positive strains of HCMV enter fibroblasts with comparable efficiency. Thus, an exclusively PC-independent pathway of infection of these cells is commonly accepted. Surprisingly, rabbit sera obtained after the application of PC-positive DBs showed higher neutralization capacity against infection of HFFs with the PC-positive TB40/E strain than did PC-negative sera (see Fig. 4). The fact that no difference was found when HFFs were infected with the PC-negative HCMV strain Towne indicates that the antibodies mediating this effect are directed against the PC. These results are remarkable, as they raise the issue of whether the PC also contributes to entry of cell-free HCMV into fibroblasts. While, in principle, steric hindrance of virus-fibroblast interactions by PC-bound antibodies or complement-mediated disruption of the virion could explain the observed findings, there is increasing evidence favoring a contribution of the pentamer to infection of fibroblasts. Overexpression of the gH/gL/gO trimer in HFFs could not completely block HCMV infection by interference, whereas overexpression of the PC in ARPE-19 cells completely blocked HCMV infection (56). Moreover, a recent study found that a combination of antibodies against the trimer and the pentamer inhibited the PC-positive strain VR1814 more effectively than did anti-trimer antibodies alone, and the authors provided data suggesting that a pentamer-mediated activation of the ErbB pathway might be involved (57). Finally, NRP-2 was recently identified as a cellular receptor of the pentamer, and this receptor is also expressed on fibroblasts (58). Both soluble NRP-2 and antibodies against NRP-2 caused a slight reduction in HCMV infection on MRC5 cells, indicating a minor contribution of the pentamer also to infection of fibroblasts. Taken together, these reports are consistent with our finding concerning a previously unnoticed role of the pentamer for entry of PC-positive HCMV strains into fibroblasts.

Towne-UL130rep-ΔGFP appears to be a suitable HCMV strain for the development of a DB production process. Human fibroblasts are the only culture system described so far that supports DB production. Clinical isolates initially express the PC when passaged on fibroblast cultures, yet most of these strains lose PC expression due to a mutation in one of the genes of the UL128-131 locus (39, 40). They, in addition, synthesize very limited amounts of DBs. We thus chose to repair the laboratory strain Towne by inserting the UL130 gene of TB40/E into Towne BAC, thereby replacing the mutated Towne gene. The expression level of the PC in TB40/E-infected fibroblasts appears to be moderate, thus resulting in its retention upon multiple passages (39) (C. Sinzger, unpublished data). In accordance with this, we did not observe a loss of PC expression following passaging of Towne-UL130rep on fibroblasts. Thus, Towne-UL130rep is suitably stable for DB production purposes. The deletion of the gene encoding GFP, however, appeared to be mandatory to exclude possible adverse effects by the application of DBs containing this protein. Consequently, the establishment of Towne-UL130rep-ΔGFP now provides a seed strain appropriate for the establishment of a downstream production process for a DB-based vaccine to be tested in clinical studies.

In conclusion, this work shows that DBs containing the PC are suitable to induce a distinct neutralizing antibody response against HCMV infection. Antibody responses against these particles may, on the other hand, be instructive in future studies to more closely define the mechanisms of entry of HCMV into different cell types.

MATERIALS AND METHODS

Cells, BAC cloning, and viruses. Primary human foreskin fibroblasts (HFFs) were cultured as described before (59). Retinal pigment epithelial cells (ARPE-19) were obtained from the ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM) plus Ham's F12 medium (1:1), supplemented with 10% (vol/vol) fetal calf serum. Human umbilical vein endothelial cells (HUVECs), conditionally immortalized with tetracycline-dependent expression of the SV40 large-T antigen and human telomerase reverse transcriptase (hTERT) (HEC-LTT), were cultured as described previously (43, 44). For growth, HEC-LTTs were cultured in endothelial cell growth medium (EGM BulletKit; Lonza) supplemented with 2 mg/ml doxycycline (AppliChem); for neutralization assays, doxycycline was omitted. All HCMV strains used in this analysis were derived from bacterial artificial chromosome (BAC) clones. HB5, a derivative of the laboratory strain AD169, was obtained from Borst et al. (60). TB40/E-BAC_{KL7} (here denoted TB40/E) is a derivative of the endotheliotropic strain TB40/E (61) that carries an intact UL US-gene region (62). TB40-BAC4-IE-GLuc is a derivative of TB40/E expressing the *Gaussia* luciferase under the HCMV IE promoter at an ectopic position (41). The Towne strain was reconstituted from Towne BAC (37, 63), kindly provided by Edward Mocarski. The Towne BAC was originally cloned from a plaque-purified derivative of the Towne strain (ATCC; VR-977). The nucleotide sequence of Towne BAC was established by Murphy et al. (64).

The generation of the UL130 repaired version of the Towne BAC strain (Towne-UL130rep) was performed by the *galk* positive/negative selection method as described by Warming and colleagues (38) and detailed for HCMV BAC mutagenesis by Mersseman and colleagues (33). In a first step, the *galk* gene was amplified from pgalk (38). The primers used for this contained 50 nucleotides homologous to the UL130 locus of HCMV, thus allowing replacement of the mutated UL130 gene in Towne BAC by *galk*. Clones obtained by this were verified by colony PCR analysis. In a second step, the *galk* gene was replaced by the wild-type (wt) UL130 gene, amplified from TB40/E-BAC_{KL7}. The UL130-specific primers used for this were flanked by the same 50 nucleotides that had been used for *galk* insertion into Towne BAC. This allowed for seamless insertion of the wt UL130, thereby replacing *galk*. After isolation of positive colonies, following negative selection for *galk* expression using desoxygalactose, the correct replacement was again verified by colony PCR. The BAC DNA of individual clones was then further characterized by restriction endonuclease digestion and agarose gel electrophoresis, as well as by DNA sequence analysis.

Virus reconstitution was achieved by transfecting of BAC DNA into HFFs. BACmid DNA for transfection was obtained from *Escherichia coli* using the Plasmid purification kit (Macherey & Nagel, Düren, Germany) according to the manufacturer's instructions. Transfections into HFFs were performed using the SuperFect transfection reagent (Qiagen, Hilden, Germany). HFFs were seeded on 6-well plates at a density of 1×10^5 cells/well using different BAC DNA concentrations. Cells were subsequently passaged until plaques became visible. The infectious supernatant was then transferred to uninfected cells for passaging of the virus. All HCMV strains were propagated on HFFs. Viral stocks were obtained by collecting the culture supernatants from infected HFFs, followed by low-speed centrifugation to remove cell debris. Supernatants were frozen at -80°C until further use.

Virus titers were determined by staining for the expression of the immediate early 1 protein (IE1) ppUL123, using monoclonal antibody p63-27 (65), kindly provided by William Britt. For this, 5×10^3 HFF or ARPE-19 cells were seeded in each well of a 96-well plate. The following day, virus stocks were diluted to 10^{-3} and 10^{-4} in culture medium and were added to the cells in octuplet replicates. Cells were fixed after 48 h for 20 min using 96% ethanol. The primary antibody p63-27 was added for 1 h in a humidified chamber at 37°C . Detection was performed by adding an anti-mouse IgG, coupled to horseradish peroxidase (HRP) (rabbit-anti-mouse immunoglobulin HRP; Dako, Hamburg, Germany) at a dilution of 1:500 for 1 h and by subsequent staining with 3-amino-9-ethyl-carbazol (AEC)- H_2O_2 for another hour. IE1-positive cells were counted, and titers were determined as the means of octuplet values.

Indirect immunofluorescence analysis and immunoblotting. Indirect immunofluorescence and immunoblot analyses were performed as previously published (66). Expression of pUL128 and pUL130 on viral particles was analyzed by immunoblotting, using gradient purified material. Three micrograms of virions and DBs was loaded per slot on a 4 to 12% Bis-Tris-polyacrylamide gel. After transfer to a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL; Millipore, Billerica, MA, USA), proteins were detected using specific antibodies. The following antibodies were used for detection: for pUL128, 4B10 (undiluted), and for pUL130, 3C5 (1:250 dilution), both kindly provided by Thomas Shenk (15); and gB:27.287 (undiluted), kindly provided by William Britt.

Quantitative PCR analysis. Viral DNA replication and release of viral DNA into the culture supernatant were performed as described before (59), using an Applied Biosystems 7500 real-time PCR system (Life Technologies GmbH, Darmstadt, Germany). Extraction of viral DNA from infected cells or culture supernatant was performed using the High Pure viral nucleic acid kits (Roche, Mannheim, Germany).

Immunizations. Immunizations of rabbits were performed by Eurogentec according to all Federation for Laboratory Animal Science Associations (FELASA) recommendations (<https://secure.eurogentec.com/product/research-animal-care.html>). Mouse immunizations were performed at the Translational Animal Research Center of the Johannes Gutenberg-University Mainz according to national and international regulations for animal welfare. Approval for the mouse immunization experiments was obtained from the local regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Microneutralization assays. A microneutralization assay, adapted from a publication by Andreoni and colleagues (67), was used for the experiments shown in Fig. 2 and 5. Serum samples were heated at 56°C for 30 min for complement inactivation. Twofold serial dilutions were prepared and added to an

equal volume of virus. Virus and serum were incubated for 4 h at 37°C in a 5% CO₂ atmosphere. HFFs were seeded on 96-well flat-bottom culture plates (BD Falcon, Heidelberg, Germany) at a density of 1.5×10^4 cells/well in minimal essential medium (MEM) containing 5% fetal calf serum (FCS) per well for 15 min prior to the addition of the virus-antibody mixture. ARPE-19 cells were seeded at the same density in 96-well plates that had been pretreated with 0.1% gelatin, using DMEM-Ham's F12 medium and 10% FCS. In this case, the virus-antibody mixture was added 3 h after seeding of the cells. Incubation with the cells was for 24 h at 37°C and 5% CO₂ for both HFF and ARPE-19 cells. After that, the supernatants were removed and the cells stained for IE1 expression, using either the antibody p63-27 and horseradish peroxidase (HRP)-coupled secondary antibodies (Plachter laboratory) or E13 and Cy3-coupled secondary antibodies (65) (Sinzger laboratory). The number of positive cells was counted in quadruple replicates. Each of the four values was divided by the mean value calculated from quadruple replicates of the positive control (no serum added) and defined as residual infectivity. The mean of these four residual infectivity values was determined for each serum dilution. The 50% neutralization titer (NT₅₀) was defined as the serum dilution that resulted in an infectivity of 50% or less (i.e., neutralization of 50% or more of infectious virus).

Gaussia luciferase-based neutralization assay. The reporter virus TB40-BAC4-IE-GLuc containing supernatant was purified as previously published (41). Two days prior to the experiment, cells were seeded on a 96 well-plate at a density of 1.5×10^4 cells per well (HFFs) or 1.2×10^4 cells/well (HEC-LTTs). For HEC-LTTs, plates were coated with 0.1% gelatin (Sigma-Aldrich). HFFs were seeded in MEM supplemented with 5% fetal bovine serum (MEM5; PAN-Biotech), GlutaMAX (Life Technologies), and 100 µg/ml gentamicin (Sigma-Aldrich). HEC-LTTs were seeded in endothelial growth medium (EGM) without doxycycline. For the neutralization assay, rabbit serum samples were 2-fold serially diluted in MEM5, mixed with the reporter virus to achieve an MOI of 1 (as calculated for HFFs), and incubated for 2 h at 37°C to allow for neutralization. Thirty minutes prior to infection, the medium of the prepared cell cultures was exchanged with fresh MEM5, and after that, preincubation cell cultures were infected with the serum-virus mixture for an additional 2 h at 37°C. The mixture was then removed and replaced by the appropriate growth medium for the respective cell type. Infected cultures were incubated overnight to allow expression and release of *Gaussia* luciferase, and luciferase activity in the supernatants was measured the following day as previously published (41), yielding values of relative light units (RLU). All RLU values were normalized to the mean RLU value of cells treated only with virus. Normalized RLU values for the various serum dilutions thus reflected relative infection levels compared to the untreated control. To determine the neutralization capacity of the rabbit serum samples, concentrations were plotted against the relative inhibition ($1 - \text{normalized RLU}$). The serum dilution that reflected a half-maximal reduction in *Gaussia* luciferase activity was determined by nonlinear regression (SigmaPlot) and given as the NT₅₀.

ACKNOWLEDGMENTS

We are indebted to Jürgen Podlech for help with the immunization of mice. We appreciate the donation of monoclonal antibodies by William Britt and Thomas Shenk and the donation of BAC clones by Hua Zhu, Fenyong Liu, and Edward Mocarski. We thank Nick Bond for a critical review of the English language.

The work was funded by Deutsche Forschungsgemeinschaft KFO183 individual project PL 236/5-2 and individual project PL 236/7-1, Else Kröner-Fresenius-Stiftung projects 2013 A_203 (to B.P.), 2014-A45, and 2016-A126 (to C.S.), ForTra gGmbH, Else Kröner-Fresenius-Stiftung, project 2017_T10 (to B.P.), and Wilhelm Sander Foundation project 2016.115.1 (to B.P.).

We report no conflicts of interest.

C.L., C.S., C.Z., P.G., and B.P. designed the study. C.L., N.B., J.J.F., C.Z., and I.P. performed the experiments. C.L., C.S., P.G., and B.P. wrote the manuscript.

REFERENCES

- Boppana SB, Britt WJ. 2013. Synopsis of clinical aspects of human cytomegalovirus disease, p 1–25. In Reddehase MJ (ed), *Cytomegaloviruses from molecular pathogenesis to intervention*, vol 2. Caister Academic Press, Norfolk, United Kingdom.
- Krause PR, Bialek SR, Boppana SB, Griffiths PD, Laughlin CA, Ljungman P, Mocarski ES, Pass RF, Read JS, Schleiss MR, Plotkin SA. 2013. Priorities for CMV vaccine development. *Vaccine* 32:4–10. <https://doi.org/10.1016/j.vaccine.2013.09.042>.
- Arvin AM, Fast P, Myers M, Plotkin S, Rabinovich R, National Vaccine Advisory Committee. 2004. Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. *Clin Infect Dis* 39:233–239. <https://doi.org/10.1086/421999>.
- Plotkin SA, Plachter B. 2013. Cytomegalovirus vaccine: on the way to the future?, p 424–449. In Reddehase MJ (ed), *Cytomegaloviruses: from molecular pathogenesis to intervention*, 2nd ed. Caister Academic Press, Norfolk, United Kingdom.
- Luisi K, Sharma M, Yu D. 2017. Development of a vaccine against cytomegalovirus infection and disease. *Curr Opin Virol* 23:23–29. <https://doi.org/10.1016/j.coviro.2017.02.003>.
- Schleiss MR. 2016. Cytomegalovirus vaccines under clinical development. *J Virus Erad* 2:198–207.
- Wang D, Fu TM. 2014. Progress on human cytomegalovirus vaccines for prevention of congenital infection and disease. *Curr Opin Virol* 6:13–23. <https://doi.org/10.1016/j.coviro.2014.02.004>.
- Plachter B. 2016. Prospects of a vaccine for the prevention of congenital cytomegalovirus disease. *Med Microbiol Immunol* 205:537–547. <https://doi.org/10.1007/s00430-016-0472-z>.
- Britt WJ, Vugler L, Butfiloski EJ, Stephens EB. 1990. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. *J Virol* 64:1079–1085.
- Marshall GS, Rabalais GP, Stout GG, Waldeyer SL. 1992. Antibodies to

- recombinant-derived glycoprotein B after natural human cytomegalovirus infection correlate with neutralizing activity. *J Infect Dis* 165: 381–384. <https://doi.org/10.1093/infdis/165.2.381>.
11. Urban M, Klein M, Britt WJ, Hassfurth E, Mach M. 1996. Glycoprotein H of human cytomegalovirus is a major antigen for the neutralizing humoral immune response. *J Gen Virol* 77:1537–1547. <https://doi.org/10.1099/0022-1317-77-7-1537>.
 12. Bernstein DI, Munoz FM, Callahan ST, Rupp R, Wootton SH, Edwards KM, Turley CB, Stanberry LR, Patel SM, Mcneal MM, Pichon S, Amegashie C, Bellamy AR. 2016. Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: a randomized clinical trial. *Vaccine* 34:313–319. <https://doi.org/10.1016/j.vaccine.2015.11.056>.
 13. Pass RF, Duliege A, Boppana S, Sekulovich R, Percell S, Britt W, Burke RL. 1999. A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. *J Infect Dis* 180:970–975. <https://doi.org/10.1056/NEJMoa0804749>.
 14. Pass RF, Zhang C, Evans A, Simpson T, Andrews W, Huang ML, Corey L, Hill J, Davis E, Flanagan C, Cloud G. 2009. Vaccine prevention of maternal cytomegalovirus infection. *N Engl J Med* 360:1191–1199. <https://doi.org/10.1056/NEJMoa0804749>.
 15. Wang D, Shenk T. 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc Natl Acad Sci U S A* 102:18153–18158. <https://doi.org/10.1073/pnas.0509201102>.
 16. Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini A, Wagner M, Gallina A, Milanese G, Koszowski U, Baldanti F, Gerna G. 2004. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J Virol* 78:10023–10033. <https://doi.org/10.1128/JVI.78.18.10023-10033.2004>.
 17. Fouts AE, Chan P, Stephan JP, Vandlen R, Feierbach B. 2012. Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin. *J Virol* 86:7444–7447. <https://doi.org/10.1128/JVI.00467-12>.
 18. Gerna G, Percivalle E, Lilleri D, Lozza L, Fornara C, Hahn G, Baldanti F, Revello MG. 2005. Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8⁺ T cells. *J Gen Virol* 86: 275–284. <https://doi.org/10.1099/vir.0.80474-0>.
 19. Kabanova A, Perez L, Lilleri D, Marcandalli J, Agatic G, Becattini S, Preite S, Fuschillo D, Percivalle E, Sallusto F, Gerna G, Corti D, Lanzavecchia A. 2014. Antibody-driven design of a human cytomegalovirus gHgLpUL128L subunit vaccine that selectively elicits potent neutralizing antibodies. *Proc Natl Acad Sci U S A* 111:17965–17970. <https://doi.org/10.1073/pnas.1415310111>.
 20. Craighead JE, Kanich RE, Almeida JD. 1972. Nonviral microbodies with viral antigenicity produced in cytomegalovirus-infected cells. *J Virol* 10:766–775.
 21. Roby C, Gibson W. 1986. Characterization of phosphoproteins and protein kinase activity of virions, noninfectious enveloped particles, and dense bodies of human cytomegalovirus. *J Virol* 59:714–727.
 22. Irmieri A, Gibson W. 1983. Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* 130:118–133. [https://doi.org/10.1016/0042-6822\(83\)90122-8](https://doi.org/10.1016/0042-6822(83)90122-8).
 23. Varnum SM, Streblow DN, Monroe ME, Smith P, Auberry KJ, Pasa-Tolic L, Wang D, Camp DG, Rodland K, Wiley S, Britt W, Shenk T, Smith RD, Nelson JA. 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. *J Virol* 78:10960–10966. <https://doi.org/10.1128/JVI.78.20.10960-10966.2004>.
 24. Büscher N, Paulus C, Nevels M, Tenzer S, Plachter B. 2015. The proteome of human cytomegalovirus virions and dense bodies is conserved across different strains. *Med Microbiol Immunol* 204:285–293. <https://doi.org/10.1007/s00430-015-0397-y>.
 25. Pepperl S, Münster J, Mach M, Harris JR, Plachter B. 2000. Dense bodies of human cytomegalovirus induce both humoral and cellular immune responses in the absence of viral gene expression. *J Virol* 74:6132–6146. <https://doi.org/10.1128/jvi.74.13.6132-6146.2000>.
 26. Topilko A, Michelson S. 1994. Hyperimmediate entry of human cytomegalovirus virions and dense bodies into human fibroblasts. *Res Virol* 145:75–82. [https://doi.org/10.1016/S0923-2516\(07\)80009-4](https://doi.org/10.1016/S0923-2516(07)80009-4).
 27. Pepperl-Klindworth S, Frankenberg N, Riegler S, Plachter B. 2003. Protein delivery by subviral particles of human cytomegalovirus. *Gene Ther* 10:278–284. <https://doi.org/10.1038/sj.gt.3301879>.
 28. Becke S, Aue S, Thomas D, Schader S, Podlech J, Bopp T, Sedmak T, Wolfrum U, Plachter B, Reyda S. 2010. Optimized recombinant dense bodies of human cytomegalovirus efficiently prime virus specific lymphocytes and neutralizing antibodies without the addition of adjuvant. *Vaccine* 28:6191–6198. <https://doi.org/10.1016/j.vaccine.2010.07.016>.
 29. Cayatte C, Schneider-Ohrum K, Wang Z, Irrinki A, Nguyen N, Lu J, Nelson C, Servat E, Gemmell L, Citkowitz A, Liu Y, Hayes G, Woo J, Van Nest G, Jin H, Duke G, McCormick AL. 2013. Cytomegalovirus vaccine strain town-derived dense bodies induce broad cellular immune responses and neutralizing antibodies that prevent infection of fibroblasts and epithelial cells. *J Virol* 87:11107–11120. <https://doi.org/10.1128/JVI.01554-13>.
 30. Sauer C, Klobuch S, Herr W, Thomas S, Plachter B. 2013. Subviral dense bodies of human cytomegalovirus stimulate maturation and activation of monocyte-derived immature dendritic cells. *J Virol* 87:11287–11291. <https://doi.org/10.1128/JVI.01429-13>.
 31. Schneider-Ohrum K, Cayatte C, Liu Y, Wang Z, Irrinki A, Cataniag F, Nguyen N, Lambert S, Liu H, Aslam S, Duke G, McCarthy MP, McCormick L. 2016. Production of cytomegalovirus dense bodies by scalable bioprocess methods maintains immunogenicity and improves neutralizing antibody titers. *J Virol* 90:10133–10144. <https://doi.org/10.1128/JVI.00463-16>.
 32. Pepperl-Klindworth S, Frankenberg N, Plachter B. 2002. Development of novel vaccine strategies against human cytomegalovirus infection based on subviral particles. *J Clin Virol* 25(Suppl 2):75. [https://doi.org/10.1016/S1386-6532\(02\)00099-9](https://doi.org/10.1016/S1386-6532(02)00099-9).
 33. Mersseman V, Besold K, Reddehase MJ, Wolfrum U, Strand D, Plachter B, Reyda S. 2008. Exogenous introduction of an immunodominant peptide from the non-structural IE1 protein of human cytomegalovirus into the MHC class I presentation pathway by recombinant dense bodies. *J Gen Virol* 89:369–379. <https://doi.org/10.1099/vir.0.83380-0>.
 34. Mersseman V, Böhm V, Holtappels R, Deegen P, Wolfrum U, Plachter B, Reyda S. 2008. Refinement of strategies for the development of a human cytomegalovirus dense body vaccine. *Med Microbiol Immunol* 197: 97–107. <https://doi.org/10.1007/s00430-008-0085-2>.
 35. Wang D, Shenk T. 2005. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *J Virol* 79:10330–10338. <https://doi.org/10.1128/JVI.79.16.10330-10338.2005>.
 36. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, Dargan DJ, McGeoch DJ, Gatherer D, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. 2004. Genetic content of wild-type human cytomegalovirus. *J Gen Virol* 85:1301–1312. <https://doi.org/10.1099/vir.0.79888-0>.
 37. Dunn W, Chou C, Li H, Hai R, Patterson D, Stolz V, Zhu H, Liu F. 2003. Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* 100:14223–14228. <https://doi.org/10.1073/pnas.2334032100>.
 38. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. 2005. Simple and highly efficient BAC recombination using *galK* selection. *Nucleic Acids Res* 33:e36. <https://doi.org/10.1093/nar/gni035>.
 39. Murrell I, Tomasec P, Wilkie GS, Dargan DJ, Davison AJ, Stanton RJ. 2013. Impact of sequence variation in the UL128 locus on production of human cytomegalovirus in fibroblast and epithelial cells. *J Virol* 87: 10489–10500. <https://doi.org/10.1128/JVI.01546-13>.
 40. Murrell I, Wilkie GS, Davison AJ, Statkute E, Fielding CA, Tomasec P, Wilkinson GW, Stanton RJ. 2016. Genetic stability of bacterial artificial chromosome-derived human cytomegalovirus during culture *in vitro*. *J Virol* 90:3929–3943. <https://doi.org/10.1128/JVI.02858-15>.
 41. Falk JJ, Laib Sampaio K, Stegmann C, Lieber D, Kropff B, Mach M, Sinzger C. 2016. Generation of a Gaussia luciferase-expressing endotheliotropic cytomegalovirus for screening approaches and mutant analyses. *J Virol Methods* 235:182–189. <https://doi.org/10.1016/j.jviromet.2016.06.008>.
 42. Sinzger C, Grefte A, Plachter B, Gouw ASH, The TH, Jahn G. 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection *in-vivo*. *J Gen Virol* 76:741–750. <https://doi.org/10.1099/0022-1317-76-4-741>.
 43. May T, Butueva M, Bantner S, Markusic D, Seppen J, MacLeod RA, Weich H, Hauser H, Wirth D. 2010. Synthetic gene regulation circuits for control of cell expansion. *Tissue Eng Part A* 16:441–452. <https://doi.org/10.1089/ten.tea.2009.0184>.
 44. Lieber D, Hochdorfer D, Stoehr D, Schubert A, Lotfi R, May T, Wirth D, Sinzger C. 2015. A permanently growing human endothelial cell line supports productive infection with human cytomegalovirus under conditional cell growth arrest. *Biotechniques* 59:127–136. <https://doi.org/10.1007/s12144-000114326>.
 45. Macagno A, Bernasconi NL, Vanzetta F, Dander E, Sarasini A, Revello MG, Gerna G, Sallusto F, Lanzavecchia A. 2010. Isolation of human monoclo-

- nal antibodies that potentially neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex. *J Virol* 84:1005–1013. <https://doi.org/10.1128/JVI.01809-09>.
46. Chiuppesi F, Nguyen J, Park S, Contreras H, Kha M, Meng Z, Kaltcheva T, Iniguez A, Martinez J, La Rosa C, Wussow F, Diamond DJ. 2018. Multi-antigenic modified vaccinia virus Ankara vaccine vectors to elicit potent humoral and cellular immune responses against human cytomegalovirus in mice. *J Virol* 92:e01012-18. <https://doi.org/10.1128/JVI.01012-18>.
 47. Lilleri D, Kabanova A, Lanzavecchia A, Gerna G. 2012. Antibodies against neutralization epitopes of human cytomegalovirus gH/gL/pUL128-130-131 complex and virus spreading may correlate with virus control in vivo. *J Clin Immunol* 32:1324–1331. <https://doi.org/10.1007/s10875-012-9739-3>.
 48. Lilleri D, Kabanova A, Revello MG, Percivalle E, Sarasini A, Genini E, Sallusto F, Lanzavecchia A, Corti D, Gerna G. 2013. Fetal human cytomegalovirus transmission correlates with delayed maternal antibodies to gH/gL/pUL128-130-131 complex during primary infection. *PLoS One* 8:e59863. <https://doi.org/10.1371/journal.pone.0059863>.
 49. Fu TM, An Z, Wang D. 2014. Progress on pursuit of human cytomegalovirus vaccines for prevention of congenital infection and disease. *Vaccine* 32:2525–2533. <https://doi.org/10.1016/j.vaccine.2014.03.057>.
 50. McVoy MA. 2013. Cytomegalovirus vaccines. *Clin Infect Dis* 57(Suppl 4):S196–S199. <https://doi.org/10.1093/cid/cit587>.
 51. Wang D, Freed DC, He X, Li F, Tang A, Cox KS, Dubey SA, Cole S, Medi MB, Liu Y, Xu J, Zhang ZQ, Finnefrock AC, Song L, Espeseth AS, Shiver JW, Casimiro DR, Fu TM. 2016. A replication-defective human cytomegalovirus vaccine for prevention of congenital infection. *Sci Transl Med* 8:362ra145. <https://doi.org/10.1126/scitranslmed.aaf9387>.
 52. Freed DC, Tang Q, Tang A, Li F, He X, Huang Z, Meng W, Xia L, Finnefrock AC, Durr E, Espeseth AS, Casimiro DR, Zhang N, Shiver JW, Wang D, An Z, Fu TM. 2013. Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine. *Proc Natl Acad Sci U S A* 110:E4997–E5005. <https://doi.org/10.1073/pnas.1316517110>.
 53. Chiuppesi F, Wussow F, Johnson E, Bian C, Zhuo M, Rajakumar A, Barry PA, Britt WJ, Chakraborty R, Diamond DJ. 2015. Vaccine-derived neutralizing antibodies to the human cytomegalovirus gH/gL pentamer potentially block primary cytotrophoblast infection. *J Virol* 89:11884–11898. <https://doi.org/10.1128/JVI.01701-15>.
 54. Plachter B, Sinzger C, Jahn G. 1996. Cell types involved in replication and distribution of human cytomegalovirus. *Adv Virus Res* 46:195–261. [https://doi.org/10.1016/S0065-3527\(08\)60073-1](https://doi.org/10.1016/S0065-3527(08)60073-1).
 55. Hetzeneker S, Helenius A, Krzyzaniak MA. 2016. HCMV induces macropinocytosis for host cell entry in fibroblasts. *Traffic* 17:351–368. <https://doi.org/10.1111/tra.12355>.
 56. Vanarsdall AL, Chase MC, Johnson DC. 2011. Human cytomegalovirus glycoprotein gO complexes with gH/gL, promoting interference with viral entry into human fibroblasts but not entry into epithelial cells. *J Virol* 85:11638–11645. <https://doi.org/10.1128/JVI.05659-11>.
 57. Kabanova A, Marcandalli J, Zhou T, Bianchi S, Baxa U, Tsybovsky Y, Lilleri D, Silacci-Fregni C, Foglierini M, Fernandez-Rodriguez BM, Druz A, Zhang B, Geiger R, Pagani M, Sallusto F, Kwong PD, Corti D, Lanzavecchia A, Perez L. 2016. Platelet-derived growth factor- α receptor is the cellular receptor for human cytomegalovirus gH/gLgO trimer. *Nat Microbiol* 1:16082. <https://doi.org/10.1038/nmicrobiol.2016.82>.
 58. Martinez-Martin N, Marcandalli J, Huang CS, Arthur CP, Perotti M, Foglierini M, Ho H, Dosey AM, Shriver S, Payandeh J, Leitner A, Lanzavecchia A, Perez L, Ciferri C. 2018. An unbiased screen for human cytomegalovirus identifies neuropilin-2 as a central viral receptor. *Cell* 174:1158–1171.e19. <https://doi.org/10.1016/j.cell.2018.06.028>.
 59. Besold K, Wills M, Plachter B. 2009. Immune evasion proteins gpUS2 and gpUS11 of human cytomegalovirus incompletely protect infected cells from CD8 T cell recognition. *Virology* 391:5–19. <https://doi.org/10.1016/j.virology.2009.06.004>.
 60. Borst EM, Hahn G, Koszinowski UH, Messerle M. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J Virol* 73:8320–8329.
 61. Sinzger C, Hahn G, Digel M, Katona R, Sampaio KL, Messerle M, Hengel H, Koszinowski U, Brune W, Adler B. 2008. Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E. *J Gen Virol* 89:359–368. <https://doi.org/10.1099/vir.0.83286-0>.
 62. Sampaio KL, Weyell A, Subramanian N, Wu Z, Sinzger C. 2017. A TB40/E-derived human cytomegalovirus genome with an intact US-gene region and a self-excisable BAC cassette for immunological research. *Biotechniques* 63:205–214. <https://doi.org/10.2144/000114606>.
 63. Marchini A, Liu H, Zhu H. 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. *J Virol* 75:1870–1878. <https://doi.org/10.1128/JVI.75.4.1870-1878.2001>.
 64. Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn G, Nelson JA, Myers RM, Shenk TE. 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc Natl Acad Sci U S A* 100:14976–14981. <https://doi.org/10.1073/pnas.2136652100>.
 65. Plachter B, Britt WJ, Vornhagen R, Stamminger T, Jahn G. 1993. Analysis of proteins encoded by IE-regions 1 and 2 of human cytomegalovirus using monoclonal antibodies generated against recombinant antigens. *Virology* 193:642–652. <https://doi.org/10.1006/viro.1993.1172>.
 66. Krömmelbein N, Wiebusch L, Schiedner G, Büscher N, Sauer C, Florin L, Sehn E, Wolfrum U, Plachter B. 2016. Adenovirus E1A/E1B transformed amniotic fluid cells support human cytomegalovirus replication. *Viruses* 8:E37. <https://doi.org/10.3390/v8020037>.
 67. Andreoni M, Faircloth M, Vugler L, Britt WJ. 1989. A rapid microneutralization assay for the measurement of neutralizing antibody reactive with human cytomegalovirus. *J Virol Methods* 23:157–167.
 68. Lehmann C (née Sauer). 2013. Impact of subviral particles of the human cytomegalovirus on the induction of the antiviral immune response. PhD thesis. Johannes Gutenberg University Mainz, Mainz, Germany.