# Horizontal Transmission of Cytomegalovirus in a Rhesus Model Despite High-Level, Vaccine-Elicited Neutralizing Antibody and T-Cell Responses

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The development of a vaccine to prevent congenital human cytomegalovirus (HCMV) disease is a public health priority. We tested rhesus CMV (RhCMV) prototypes of HCMV vaccine candidates in a seronegative macaque oral challenge model. Immunogens included a recombinant pentameric complex (PC; gH/gL/pUL128/pUL130/pUL131A), a postfusion gB ectodomain, and a DNA plasmid that encodes pp65-2. Immunization with QS21-adjuvanted PC alone or with the other immunogens elicited neutralizing titers comparable to those elicited by RhCMV infection. Similarly, immunization with all 3 immunogens elicited pp65-specific cytotoxic T-cell responses comparable to those elicited by RhCMV infection. RhCMV readily infected immunized animals and was detected in saliva, blood, and urine after challenge in quantities similar to those in placebo-immunized animals. If HCMV evades vaccine-elicited immunity in humans as RhCMV evaded immunity in macaques, a HCMV vaccine must elicit immunity superior to, or different from, that elicited by the prototype RhCMV vaccine to block horizontal transmission.

**Keywords.** cytomegalovirus; vaccine; neutralizing antibody; protective efficacy; rhesus macaque; RhCMV.

Human cytomegalovirus (HCMV) infection of immune-competent individuals generally manifests with an acute phase followed by a life-long latent phase and is often asymptomatic. However, new infection or reactivation from latent HCMV infection in immunosuppressed patients, such as transplant recipients or those with AIDS, can cause serious disease and even death. Primary HCMV infection of a seronegative pregnant woman results in transmission to her fetus in approximately 30% of cases [1, 2]. Approximately 10% of congenitally infected infants will have symptoms at birth, with approximately 30%–40% of those exhibiting severe disease, which can include hearing loss, vision loss, microcephaly, and developmental delay. Nonprimary infection of a seropositive pregnant woman or reactivation of her latent HCMV also can result in transplacental transmission. The probability of transmission to the fetus and severe sequelae may be substantially lower after nonprimary maternal infection, though studies of the outcomes of nonprimary maternal infection

are inconsistent [1, 2]. A prophylactic HCMV vaccine is urgently needed to prevent congenital HCMV disease, as well as HCMV disease in immunosuppressed patients.

At least 3 viral glycoproteins or glycoprotein complexes mediate HCMV entry into cells: glycoprotein B (gB), which is required for CMV entry into all target cells [3]; the gH/gL/pUL128/pUL130/pUL131 heteropentamer complex (PC) [3], which is important for entry into epithelial and endothelial cells; and gH/gL/gO, which is important for entry into fibroblasts [4–7]. The viral fusion glycoprotein, gB, transitions from a metastable prefusion conformation to a stable postfusion conformation as it fuses membranes during cell entry [8, 9]. Postfusion gB has been isolated in a well-characterized form stable enough to test its immunogenicity. Postfusion gB elicits strong nonneutralizing antibody responses, strong complement-dependent neutralizing responses, and much weaker complement-independent neutralizing responses [10, 11]. A subunit vaccine candidate containing an adjuvanted, recombinant, postfusion CMV gB antigen has demonstrated statistically significant protection from horizontal transmission of CMV to seronegative new mothers [12], and a trend toward protection of seronegative adolescent girls [13]. Although the observed level of protection was insufficient to justify further development of a postfusion gB-based vaccine, the findings suggest that mechanisms other than virus neutralization may have some protective capacity. Prefusion HCMV gB has been

stabilized for structure determination [14], but there are as yet no reports of the immunogenicity of a preparation of gB that has been convincingly demonstrated to be in the prefusion conformation.

The PC is the dominant target for neutralizing antibody (nAb) responses in natural HCMV infections [9, 15], and sera from mice and monkeys immunized with recombinant PC neutralize HCMV infection of epithelial and endothelial cells and fibroblasts to high titers [16–19]. Directly testing the protective efficacy of HCMV antigens in animal models is not feasible because HCMV only infects humans. Infection of specific-pathogen–free (SPF) rhesus macaques by RhCMV is a rigorous animal model for CMV pathogenesis and vaccine efficacy [20]. Vaccination of rhesus macaques with modified vaccinia virus Ankara (MVA) vectors expressing soluble or membrane-associated RhCMV PC did not efficiently prevent RhCMV infection after subcutaneous challenge, but did reduce viral loads [21]. The nAb response elicited by MVA-vectored PC immunization was modest and far below responses elicited by recombinant HCMV PC immunization of mice or natural HCMV infection of humans. A nucleoside modified RNA-based vaccine candidate that encodes the human CMV PC and postfusion gB is in clinical testing [22].

Here, we report the immunogenicity of RhCMV PC and its protective efficacy against oral challenge of SPF rhesus macaques with UCD52, a virulent strain of RhCMV that has a functional PC [23]. In addition, we tested the protective efficacy of immunization with RhCMV PC together with 2 other RhCMV immunogens—postfusion gB and DNA that encodes RhCMV pp65-2, an orthologue of the HCMV pp65 tegument protein.

## METHODS

### Vaccine Immunogens

#### Expression and Purification of Recombinant RhCMV UCD52 PC

The PC was expressed in SF9 insect cells from a single baculovirus with the RedVax GmbH platform design, which is based on the Multibac system [24, 25]. The 5 coding sequences (accession numbers: HQ667932-gH; HQ667933-gL; GU552456-UL128, -UL130, -UL131a) were synthesized with codon optimization for insect cell expression. Glycoprotein H is the only protein in the PC with a transmembrane domain, and its coding sequence was truncated to express a soluble ectodomain with a C-terminus at amino acid 695. The recombinant RhCMV strain UCD52 [26] PC was isolated from 12 L of SF9 culture medium using affinity chromatography with Ni Sepharose (GE Healthcare) followed by cation exchange chromatography with Toyopearl-SP 650M (Tosoh Bioscience). The final preparation of PC was quantified by micro-BCA (Invitrogen) and characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

#### Production of RhCMV gB674

The N-terminal 674 residues of UCD52 gB (GenBank accession ADD92468), named gB674, with a C-terminal 6×His tag were expressed as a soluble ectodomain in Expi293F cells (Invitrogen) by transient transfection. Five liters of HEK293 conditioned culture medium was concentrated 14× and 3× diafiltered into binding buffer (20 mM NaPO$_4$, 300 mM NaCl, pH 7.4) by 10 kDa molecular weight cutoff tangential flow filtration. The diafiltrate was applied to a 5-cm × 17-cm Ni Sepharose 6 FF column (GE Healthcare) and washed with 20 mM NaPO$_4$, 150 mM NaCl, 0.14% Triton X-100, pH 6.8–7, followed by 20 mM NaPO$_4$, 300 mM NaCl, 100 mM imidazole, pH 6.8–7. gB674 was eluted in 20 mM NaPO$_4$, 150 mM NaCl, 500 mM imidazole, pH 6.8–7. The pooled fractions were concentrated to 45 mL by tangential flow filtration and applied to a Superdex 200 pg (Cytiva) size-exclusion column (5 cm × 55 cm) in 20 mM NaPO$_4$, 150 mM NaCl, pH 6.8–7. The purification yielded 300 mg of pure gB674 protein.

#### UCD52 pp65-2 DNA

The coding sequence of RhCMV UCD52 pp65-2 (GenBank accession QQL11309) was codon-optimized for mammalian cell expression and cloned into an expression cassette pJV7563 as previously described [27]. The plasmid was expanded in BD1175 host cells (a noncommercial product of Pfizer, Inc) and purified by anion exchange chromatography (Fractogel TMAE [M] resin; EMD Millipore). Isoforms of the purified plasmid were analyzed by anion exchange high-performance liquid chromatography using a CIMac pDNA-0.3 analytical column. The final product contained 89% supercoiled DNA and was passed through a 0.22-μm filter.

### RhCMV Strain and Monkey Cells

RhCMV strain UCD52, primary monkey kidney epithelial cells (MKE) and telomerized rhesus fibroblast cells (Telo-RF) were prepared as described [28–30]. UCD52 viral stock for animal challenge was passaged 4 times on MKE cells and expanded once on Telo-RF cells. Virus stocks were titrated on MKE or Telo-RF cells using a single-round entry assay in which virus-infected cells were enumerated by immunostaining for immediate-early-1 (IE1) 48 hours after virus inoculation.

### Vaccination and Challenge Studies in SPF Rhesus Macaques

SPF rhesus macaques (*Macaca mulatta*) were bred and maintained at the California National Primate Research Center as reported [31]. All animal protocols were approved in advance by the Institutional Animal Care and Use Committees of the University of California, Davis (UC Davis) and Pfizer. UC Davis is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animals were seronegative for RhCMV prior to immunization or inoculation. The animals were 12–24 months of age. About half

were male, and half were female. The protein antigens and pp65-2 DNA used for vaccination tested negative for adventitious agents and had endotoxin levels below the maximum permissible.

Groups of 6 rhesus macaques were injected intramuscularly (IM) with the protein immunogens adjuvanted with QS-21 3 times at 4-week intervals. One group was immunized with pp65-2 DNA by electroporation in 1 limb and with the protein immunogens in the contralateral limb. For DNA vaccination, 2 mg of plasmid was formulated in 0.5 mL phosphate-buffered saline and delivered into the quadriceps muscle using the TriGrid Delivery System by Ichor Medical Systems following the manufacturer's protocol (Ichor Medical Systems). A group of 6 animals was mock vaccinated by electroporation as control.

Two weeks after the third vaccination, the vaccine- or placebo-immunized animals were challenged by 5 weekly inoculations of $8 \times 10^5$ plaque-forming units (pfu) of RhCMV UCD52 (GenBank accession MT157339) into the buccal pouch, as previously reported [32]. Samples of blood (EDTA-treated plasma and peripheral blood mononuclear cells [PBMCs]), saliva (oral swab), and urine (cystocentesis complemented by pan catch when needed) were collected by conventional methods and used for serological and virological testing using previously described methods with some modifications [26, 33–36].

## RESULTS

### Expression and Characterization of Recombinant UCD52 PC

The PC was purified by affinity chromatography from recombinant baculovirus-infected insect cells by nickel affinity chromatography using the C-terminal histidine tag appended to gH, followed by cation exchange chromatography. SDS-PAGE under reducing and nonreducing conditions produced a pattern of bands similar to that of the HCMV PC published by others (Figure 1) [37]. We confirmed the identities of the major resolved bands to be gH, gL, pUL128, pUL130, and pUL131A by liquid chromatography with tandem mass spectrometry (LC-MS/MS; Supplementary Table 1).

### Expression and Characterization of Recombinant, Postfusion gB674

Guided by X-ray crystal structures of HCMV postfusion gB [8, 9], we truncated RhCMV gB (GenBank Accession GU552457) at residue 674 to produce a soluble, recombinant, trimeric ectodomain. The resulting construct, gB674, includes the signal peptide, mutation of the furin recognition motif (from $_{429}$RRKR$_{432}$ to ATKA), 3 mutations (F128G, I129H, and W213A) in the 2 fusion loops to prevent aggregation, and a GSG linker to a C-terminal 6×His purification tag (Figure 2A). After expression in mammalian cells and purification, reducing SDS-PAGE analysis of gB674 produced a single, uniform monomer band with a migration around 100–130 kDa, which is higher than the approximately 76 kDa of the predicted molecular weight of the
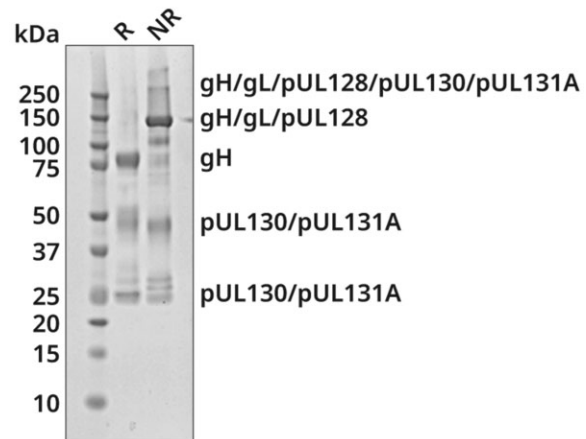


**Figure 1.** Expression and characterization of recombinant UCD52 PC. The presence of the 5 components of the PC (gH, gL, pUL128, pUL130, and pUL131A) was confirmed by SDS-PAGE analysis using 4%–12% bis-tris polyacrylamide gel. The PC samples were analyzed at nonreducing (NR) and reducing (R) conditions. Protein bands were visualized with Coomassie blue staining, and the proteins corresponding to individual bands were identified by LC-MS/MS (see Supplementary Table 1). Abbreviations: LC-MS/MS, liquid chromatography with tandem mass spectrometry; PC, pentameric complex; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

polypeptide, possibly due to glycosylation (Figure 2B). By non-reducing SDS-PAGE, more slowly migrating species were seen, consistent with trimer formation and a higher-order association. By size exclusion chromatography in the presence of 1 mM EDTA, a single, homogeneous peak with an apparent molecular weight of 313 kDa was observed, consistent with a gB674 trimer (Figure 2C). The gB674 preparation had a purity of >98% by SDS-PAGE. Transmission electron microscopy after negative staining revealed monodispersed gB674 trimers with rod-like shapes approximately 17 nm in length and with 3 globular densities, consistent with postfusion HCMV gB crystal structures (Figure 2D) [8].

### Design of a DNA Immunogen Encoding UCD52 pp65-2

HCMV pp65 is a tegument phosphoprotein and a major target for cytotoxic T-cell responses during natural infection [38, 39]. RhCMV encodes 2 homologues of HCMV pp65: pp65-1 and pp65-2, the latter being the more important cytotoxic T-cell target in chronically RhCMV-infected monkeys [40]. In transiently transfected COS-7 cells, a plasmid encoding the UCD52 RhCMV pp65-2 open reading frame under control of the CMV promoter directed high-level expression of pp65-2, as detected by immunostaining of nuclei with an antibody that recognized the 6×His tag appended to the pp65-2 C-terminus (Figure 3).

### Immunogenicity of PC, gB674, and pp65 in Rhesus Macaques

We immunized 4 groups of 6 SPF rhesus macaques at weeks 0, 4, and 8: (1) buffer only (20 mM Tris, 150 mM NaCl, 6 mM
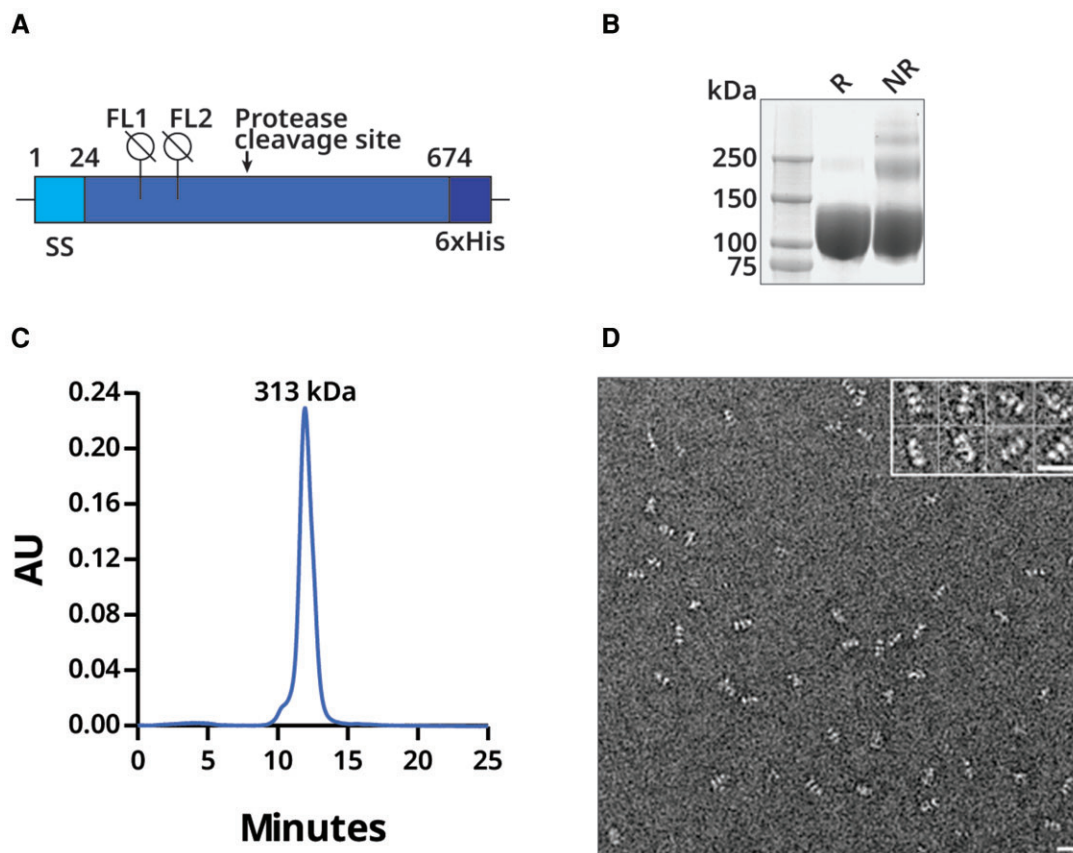
**Figure 2.** Expression and characterization of recombinant gB674 from UCD52 RhCMV. *A*, Design of gB674 [23, 24]. *B*, Coomassie-stained SDS-PAGE of purified gB674 separated under reducing (R) or nonreducing (NR) conditions. *C*, Size exclusion chromatography analysis of gB674. *D*, Electron microscopy of negatively stained gB674 at a magnification of 1:50 000. The white bar represents 20 nm, and the inset shows a subset of the particles. Abbreviations: 6×His, 6-histidine affinity tag; FL, fusion loop; gB, glycoprotein B; RhCMV, rhesus cytomegalovirus; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SS, signal sequence.

EDTA, 0.1% PS80, pH 7.7); (2) PC; (3) PC and gB674; or (4) PC, gB674, and pp65-2 DNA. QS-21 adjuvant (50 µg/0.5 mL) was included in all groups. Protein antigens were delivered by IM injection in 1 arm, and the pp65-encoding DNA was delivered to the contralateral arm by electroporation (Figure 4). Prior to immunization, all animals were confirmed to be seronegative for RhCMV gB-binding IgG. We collected plasma, PMBCs, saliva, and urine at the indicated timepoints (Figure 4).

Plasma neutralizing titers against UCD52 were measured without complement on Telo-RF (fibroblast) and MKE (epithelial) target cells (Figure 5A and 5B). Neutralizing titers in animals immunized with PC alone or with PC and gB674 were undetectable 4 weeks after the first dose, and were modestly higher than the geometric mean of the neutralizing titers from 20 naturally infected adult rhesus macaques 2 weeks after the second and third doses, respectively, when the animals were challenged with RhCMV. Neutralizing titers were similar 2 weeks following the second and third doses, as were neutralizing titers measured on fibroblasts or on epithelial cells. Notably, none of the 6 control animals had detectable RhCMV

neutralizing titers. All 6 animals immunized with PC and gB674 developed antibodies that bound a tagless version of the gB antigen, detectable in week 10 (2 weeks after dose 3) plasma diluted 1:100 000 (Figure 5C). The control animals remained negative for gB-binding antibodies prior to virus challenge.

By week 6 (2 weeks after dose 2), PBMCs from all 6 animals immunized with PC, gB674, and pp65-encoding DNA responded to stimulation with a pp65 peptide pool by ELISPOT, with approximately 1000 cells secreting interferon-γ (IFN-γ) per $1 \times 10^6$ PBMC (Figure 5D). This level of response is similar to that of PBMCs from naturally infected rhesus macaques (data not shown). In the placebo group, approximately 2 or fewer cells per $1 \times 10^6$ PBMC obtained before RhCMV challenge secreted IFN-γ after stimulation with the peptides (Figure 5D).

### Protection from RhCMV Challenge
The macaques were orally challenged with $8 \times 10^5$ pfu of RhCMV UCD52 weekly for 5 weeks (weeks 10–14) starting
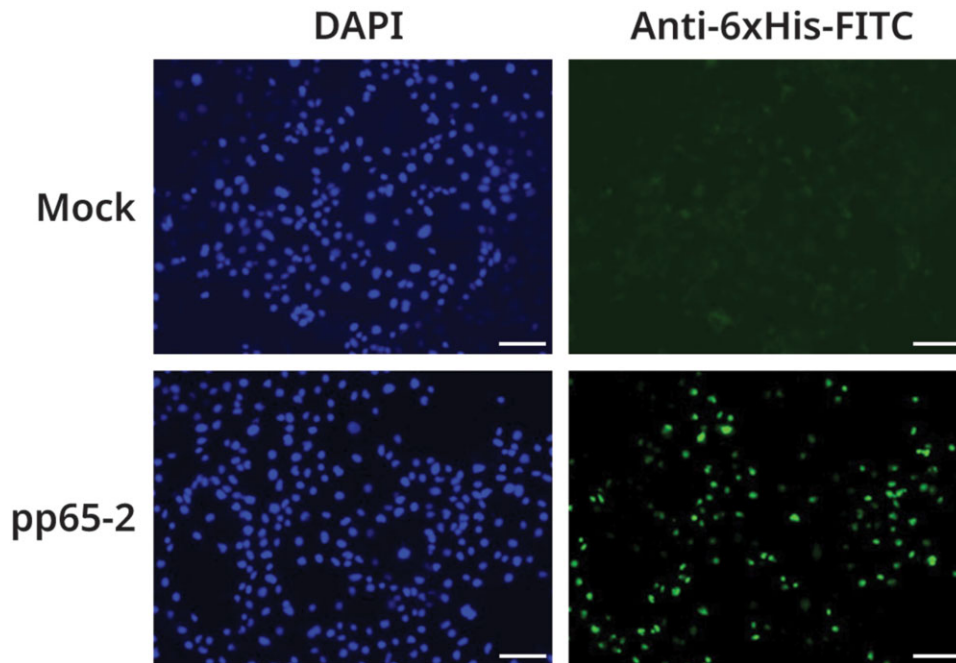
**Figure 3.** Fluorescence microscopy of RhCMV UCD52 pp65-2 expressed from a transfected DNA vaccine vector. An anti-6×His antibody conjugated with FITC (green) was used to assay the presence of histidine-tagged UCD52 pp65-2 in mock transfected (right upper panel) or DNA vaccine vector transfected (right lower panel) COS-7 cells. Nuclei of the same cells were stained with DAPI (blue in the left panels). Scale bar = 100 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; RhCMV, rhesus cytomegalovirus.

2 weeks after the third vaccine dose (Figure 4). Infection was detected by seroconversion to UCD52 viral interleukin 10 (vIL-10; a RhCMV antigen not used for immunization) and by detection of UCD52 DNA by quantitative polymerase chain reaction (qPCR). Following oral UCD52 challenge, 5 of 6 animals in the control-immunized group and in each of the 3 vaccinated groups developed vIL-10–binding antibody responses and had detectable viral DNA in plasma, saliva, or urine (Figure 6 and Figure 7). We did not observe significant differences in the gB-binding IgG levels and neutralization titers between the non-seroconverted and seroconverted animals within the same group. Detection of viral DNA was intermittent in some animals. Overall, similar quantities of viral DNA were detected after challenge in animals immunized with the RhCMV antigens and in those immunized with the control preparation.

## DISCUSSION

Immunization of women and adolescent girls with an adjuvanted postfusion gB subunit vaccine provides partial, transient protection from horizontal transmission of HCMV, despite the modest neutralizing titers elicited by this immunogen [12, 13]. Because the PC elicits much higher neutralizing titers than does postfusion gB, and pp65 elicits cytotoxic T cells, it is possible that adding a PC antigen (and potentially a pp65 antigen) to a gB-containing vaccine could provide a much greater level and duration of protection. On the other hand, the ability of HCMV to cause repeated infections—even in those who are already chronically infected and have both neutralizing antibodies and T cells that recognize HCMV [1]—raises questions about whether such immunization can prevent HCMV disease.
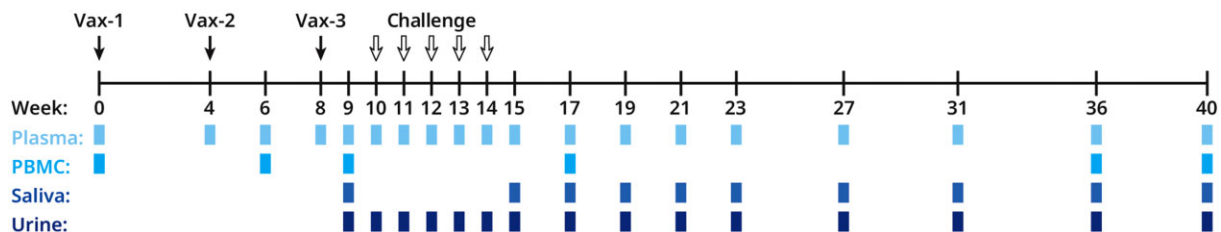


**Figure 4.** Vaccination, challenge, and sample collection timeline. Vaccinations denoted with solid black arrows, oral virus challenges denoted with hollow black arrows, the indicated sample collections denoted with colored boxes. Abbreviations: PBMC, peripheral blood mononuclear cell; Vax, vaccination.
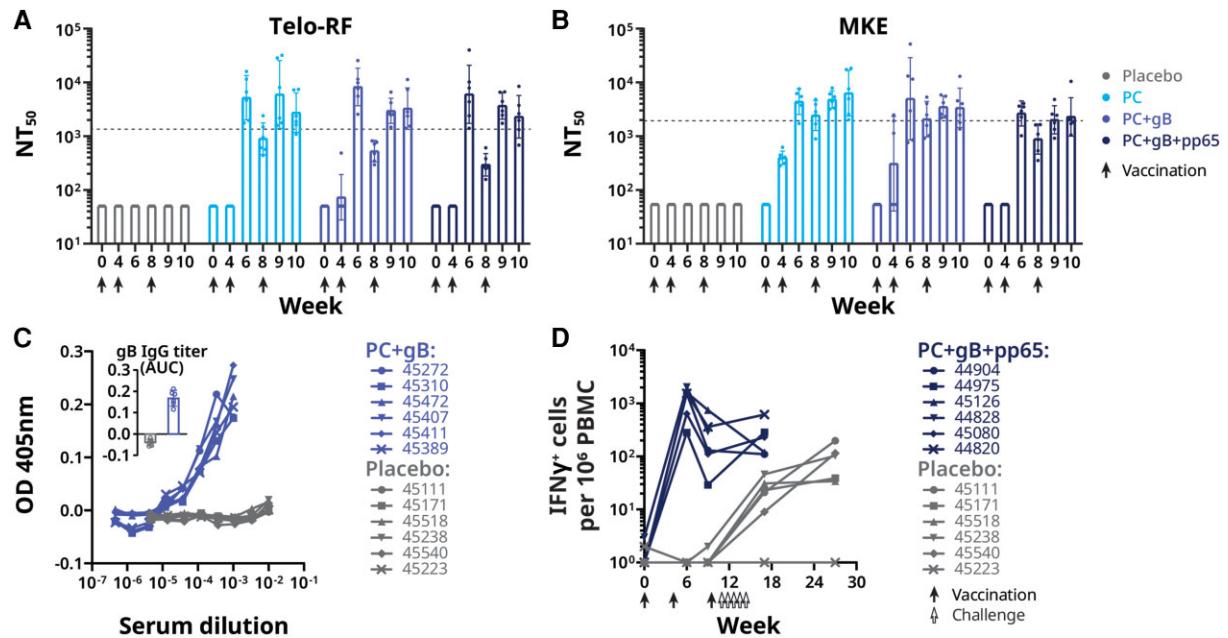
**Figure 5.** Immune responses elicited by immunization of rhesus macaques with QS-21 and placebo, PC, gB674, and pp65-encoding DNA. *A* and *B*, RhCMV neutralizing responses in plasma samples drawn at the indicated time points as measure on Telo-RF (*A*) and MKE (*B*). Geometric mean of $NT_{50}$ from 20 naturally RhCMV-infected rhesus macaques are indicated by the dotted lines. *C*, RhCMV gB-binding IgG responses in immunized rhesus macaques measured by ELISA (OD 405 nm; inset, AUC). *D*, IFN-γ ELISPOT responses to pp65-2 DNA vaccination in PBMCs obtained at the indicated times points. Frequency of RhCMV pp65-2–specific IFN-γ–responding cells was enumerated to IFN-γ$^+$ cells per $1 \times 10^6$ PBMCs. Each symbol represents the mean of technical triplicates from individual animals. Each animal is represented by its ID number. Abbreviations: AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; gB, glycoprotein B; IFN-γ, interferon-γ; IgG, immunoglobulin G; MKE, primary monkey kidney epithelial cell; $NT_{50}$, mean neutralizing titer; OD, optical density; PBMCs, peripheral blood mononuclear cells; RhCMV, rhesus cytomegalovirus; Telo-RF, transformed fibroblast.

Therefore, we sought a preclinical proof-of-concept before advancing an HCMV vaccine to clinical testing.

We tested whether a prototype vaccine containing RhCMV postfusion gB and PC with QS-21 adjuvant and pp65-encoding DNA could protect rhesus macaques from oral challenge with RhCMV. QS-21 is a potent vaccine adjuvant that enhances both

antibody and cell-mediated responses and is in the formulation of a widely used licensed vaccine [41]. We delivered the RhCMV challenge orally rather than by injection because mucosal exposure is arguably a more relevant model for the horizontal transmission of HCMV in human populations [23]. We chose UCD52 as the challenge strain because, unlike the related
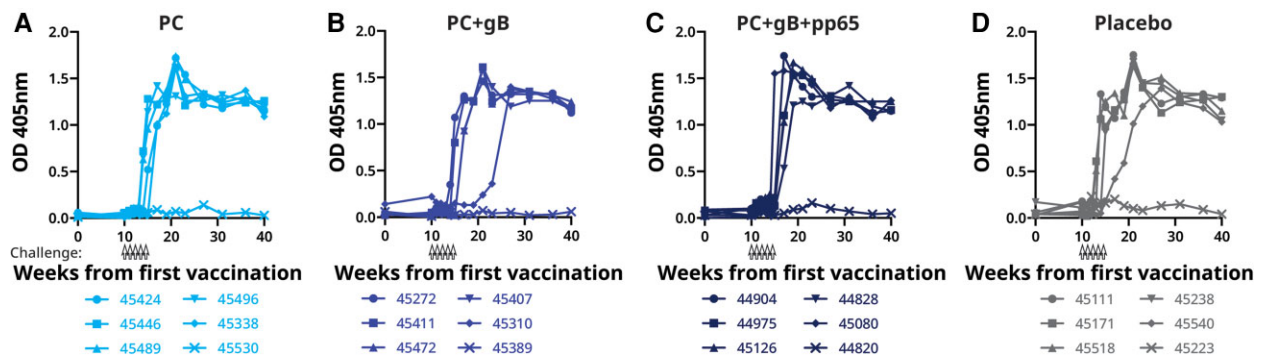


**Figure 6.** RhCMV vIL-10–binding IgG antibody in immunized and RhCMV strain UCD52-challenged rhesus macaques. *A–D*, ELISA results from the groups of animals immunized with PC, PC+gB, PC+gB+pp65 and placebo at various time points. Oral inoculations with RhCMV are indicated by hollow black arrows. The presence of anti–vIL-10 antibody was determined by ELISA. Because RhCMV vIL-10 is not in the vaccine preparations, acquisition of vIL-10–binding antibody is a biomarker of virus acquisition. Absorbance values >0.3 at OD 405 nm indicate seroconversion. Each animal is represented by its ID number. Abbreviations: ELISA, enzyme-linked immunosorbent assay; gB, glycoprotein B; IgG, immunoglobulin G; OD, optical density; PC, pentameric complex; RhCMV, rhesus cytomegalovirus; vIL-10, viral interleukin 10.
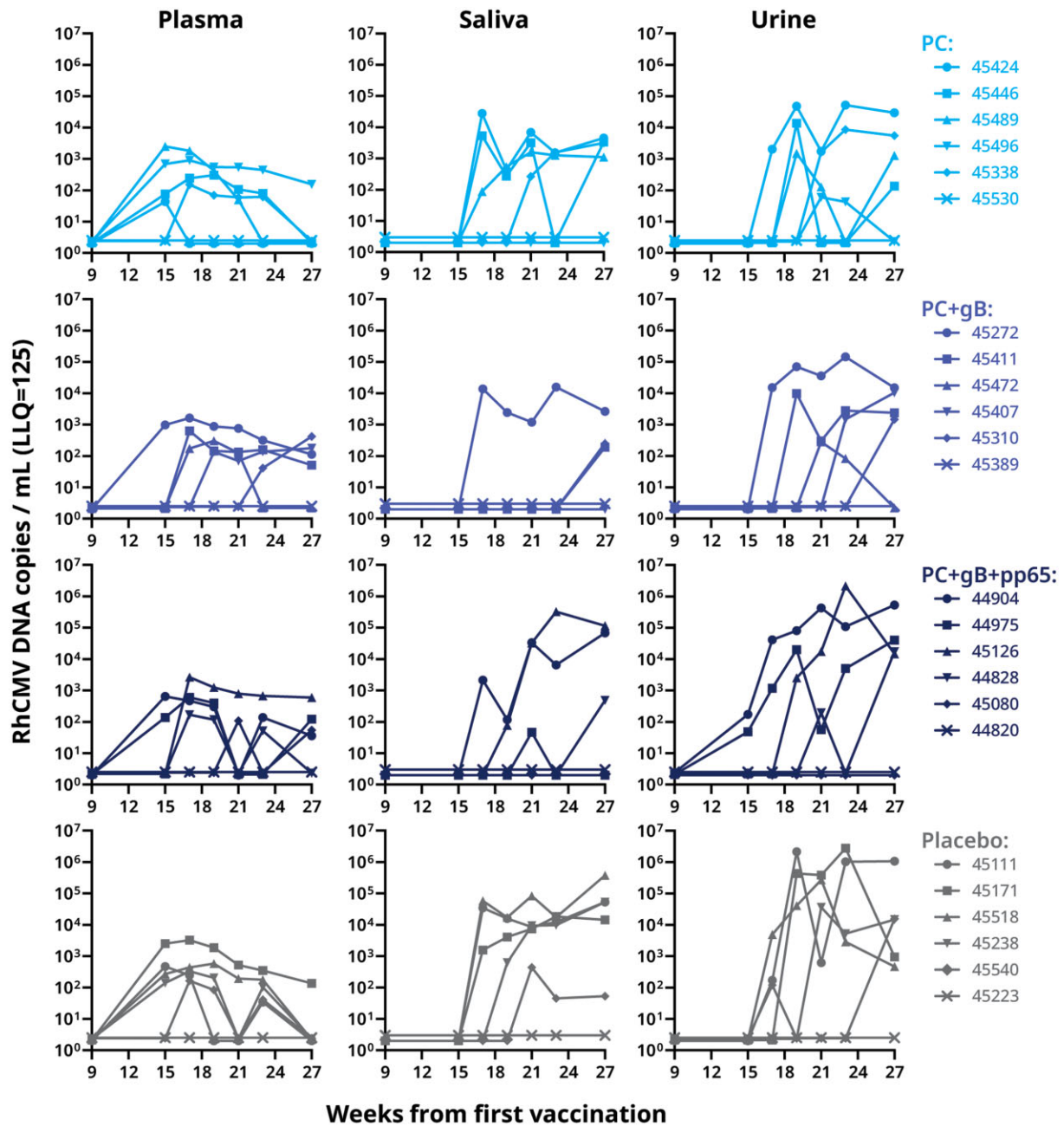
**Figure 7.** RhCMV DNA detection by qPCR in plasma, saliva, and urine samples from immunized and RhCMV-challenged rhesus macaques. RhCMV was inoculated weekly during weeks 10–14. Each animal is represented by its ID number. Abbreviations: gB, glycoprotein B; LLQ, lower limit of quantification; PC, pentameric complex; qPCR, quantitative polymerase chain reaction; RhCMV, rhesus cytomegalovirus.

strain UCD59, UCD52 contains a functional PC [29, 42]. Immunization of the animals 3 times with preparations that contained PC elicited neutralizing titers as high, or higher than, those elicited by natural RhCMV infection. Moreover, adding postfusion gB elicited strong gB-binding antibodies, and adding electroporated DNA encoding RhCMV pp65 elicited strong T-cell responses that recognized RhCMV pp65 peptides. However, these immune responses did not prevent RhCMV infection after oral inoculation, did not prevent spread to the

kidney or the genitourinary tract (as evidenced by viral DNA in the urine), and did not consistently reduce the amount or duration of viral shedding. This failure of protection is reproducible, as we had obtained similar results in a previous study in the same UCD52 challenge model after immunization with a heterologous UCD59 PC antigen, which elicited similar RhCMV neutralizing titers to those observed in the current study (Supplementary Figures 1 and 2). These findings demonstrate the ability of RhCMV to evade vaccine-elicited immunity.

CMV has several immune evasion mechanisms, and infection at mucosal surfaces is difficult to prevent. Once CMV infects at a mucosal surface, it may spread cell to cell and be carried in the bloodstream inside infected cells [43]. More than 40 gene products of the HCMV genome are believed to have immune evasion functions [44]. To protect against CMV, it may be necessary not only to elicit high titers of neutralizing antibodies and strong $CD8^+$ T-cell responses, but also to elicit immune responses that can counter viral immune evasion and augment mucosal immunity. In addition, analysis of binding of cell surface and soluble gB by poorly neutralizing serum IgG from participants in human trials suggests that binding to prefusion gB is potentially associated with protection from horizontal transmission [45]. We recently determined the structure of prefusion gB [14] and are working to produce conformationally homogenous prefusion and postfusion preparations of CMV gB to study the properties of each conformer as antigen and immunogen. Another consideration is that the basis for protection against vertical transmission could be different, with different vaccination outcomes, as suggested by passive transfer experiments to prevent vertical transmission in $CD4^+$ T-cell–depleted pregnant rhesus macaques [46].

One limitation of this study is the small number of immunized macaques. Protection against experimental horizontal transmission was not observed with 6 seronegative macaques receiving each vaccine or placebo preparation. In a human trial of protection against natural horizontal transmission in which 234 seronegative women were immunized with MF59-adjuvanted postfusion gB and 230 received placebo, 50% protection was observed ($P = .02$) [12]; in a second trial in which 195 seronegative adolescent girls received the same vaccine and 207 received placebo, the level of protection did not reach statistical significance [13]. Thus, if the experimental RhCMV vaccine preparations prevented horizontal transmission in macaques at the modest level observed in human trials of a MF59-adjuvanted postfusion gB subunit vaccine candidate, the number of macaques would be insufficient to detect statistically significant protection.

Another limitation of this study is the need to challenge the rhesus macaques with a homologue of HCMV rather than with HCMV itself. It is possible that rhesus macaques are less amenable to vaccine-induced protection against RhCMV than humans are against HCMV. The UCD52 challenge virus contains genetic changes in UL128 and RL13 [29, 42]. Loss of the gene encoding RL13 has been associated with increased HCMV replication in cell culture in the presence of neutralizing antiserum, as well as with viral resistance to antibody-mediated cytotoxic cells [47, 48]. Although it is possible that our viral challenge regimen of 5 doses of $8 \times 10^5$ pfu per oral inoculum is greater than typical human exposures, the challenge dose does not seem excessive, with only 4 of 5 placebo-immunized macaques showing signs of RhCMV infection after challenge in this study. We previously reported that, after a single oral inoculum of $8 \times 10^5$ pfu of RhCMV strain UCD52, 3 of 4 unimmunized macaques were infected; with $8 \times 10^4$ pfu, 1 of 4 was infected; and with $8 \times 10^3$ pfu, zero of 4 were infected [23]. Moreover, $8 \times 10^5$ pfu is comparable to the range of RhCMV genome copies ($10^3$–$10^6$/mL by qPCR) persistently shed in the saliva of animals naturally exposed to endemic strains of RhCMV circulating in group-housed macaques [49]. Human children also shed large quantities of CMV in saliva [50], and multiple daily household exposures of caregivers are possible.

Because transmission of HCMV to the fetus during the first trimester is more likely to cause severe congenital disease than transmission later in pregnancy, in many cases it may be too late to start immunization with a future CMV vaccine by the time pregnancy is recognized and prenatal care is begun. Therefore, for maximal public health impact, it may be necessary to immunize before the childbearing years and achieve a duration of robust prevention lasting through the childbearing years. For these reasons, there is great value in a stringent animal model of protection from CMV infection. Partial, transient protection from CMV is unlikely to be sufficient to prevent congenital disease with real-world use. A prototype vaccine that robustly protects rhesus macaques from RhCMV challenge would provide a strong guide to the composition of a human CMV vaccine that merits clinical testing. Future studies may include further refinement of the RhCMV challenge model, and may include additional CMV vaccine antigens, such as antigens that could elicit immunity that can block HCMV immune evasion.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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