Additive Protection against Congenital Cytomegalovirus Conferred by Combined Glycoprotein B/pp65 Vaccination Using a Lymphocytic Choriomeningitis Virus Vector

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ABSTRACT
Subunit vaccines for prevention of congenital cytomegalovirus (CMV) infection based on glycoprotein B (gB) and pp65 are in clinical trials, but it is unclear whether simultaneous vaccination with both antigens enhances protection. We undertook evaluation of a novel bivalent vaccine based on nonreplicating lymphocytic choriomeningitis virus (rLCMV) vectors expressing a cytoplasmic tail-deleted gB [gB(dCt)] and full-length pp65 from human CMV in mice. Immunization with the gB(dCt) vector alone elicited a comparable gB-binding antibody response and a superior neutralizing response to that elicited by adjuvanted subunit gB. Immunization with the pp65 vector alone elicited robust T cell responses. Comparable immunogenicity of the combined gB(dCt) and pp65 vectors with the individual monovalent formulations was demonstrated. To demonstrate proof of principle for a bivalent rLCMV-based HCMV vaccine, the congenital guinea pig cytomegalovirus (GPCMV) infection model was used to compare rLCMV vectors encoding homologs of pp65 (GP83) and gB(dCt), alone and in combination versus Freund’s adjuvanted recombinant gB. Both vectors elicited significant immune responses, and no loss of gB immunogenicity was noted with the bivalent formulation. Combined vaccination with rLCMV vectored GPCMV gB(dCt) and pp65 (GP83) conferred better protection against maternal viremia than subunit or either monovalent rLCMV vaccine. The bivalent vaccine also was significantly more effective in reducing pup mortality than the monovalent vaccines. In summary, bivalent vaccines with rLCMV vectors expressing gB and pp65 elicited potent humoral and cellular responses and conferred protection in the GPCMV model. Further clinical trials of LCMV vectored HCMV vaccines are warranted.

KEYWORDS congenital cytomegalovirus, cytomegalovirus, fetal infection, live vector vaccines, lymphocytic choriomeningitis virus, placental immunology

Infection with human cytomegalovirus (HCMV) causes a considerable burden in immunocompromised patients receiving solid organ or hematopoietic stem cell transplants, HIV-infected individuals, and newborns that acquire infection in utero (1, 2). By preventing congenital HCMV infection, a preconception vaccine could provide a highly cost-effective public health advance (3). Virus-neutralizing antibody targeting viral envelope glycoproteins, as well as cellular immune responses (CD4+ and CD8+)}
targeting multiple proteins, play important roles in protection against acquisition and reactivation of infection (4–8). Recombinant vaccines based on envelope glycoprotein B (gB), expressed in mammalian cells and admixed with proprietary adjuvants, such as the squalene-based oil-in-water adjuvant MF59, have demonstrated varied degrees of protection against HCMV infection and/or disease in both immunocompetent women (9, 10) and immunocompromised solid organ transplant recipients (11).

Other expression strategies targeting HCMV proteins have been evaluated in phase I and phase II studies in healthy volunteers and, in some cases, hematopoietic stem cell transplant recipients (12–16). Two multivalent recombinant vaccine candidates have been evaluated in the clinical setting. Propagation-defective alphavirus replicon particles expressing gB and a pp65-immediate-early 1 (IE1) fusion protein were immunogenic in a phase I study, but this combination was not pursued further (15). A DNA vaccine expressing gB and pp65 has advanced through phase II trials and has shown signs of efficacy. However, the data suggest only modest immune responses to gB (12). Thus, the efficacy of a bivalent vaccine candidate that elicits both strong humoral and cellular immune responses is unknown.

Because of the species specificity of cytomegaloviruses, experimental HCMV vaccines cannot be evaluated for efficacy against congenital infection in animal models. Guinea pig cytomegalovirus (GPCMV) recapitulates the pathogenesis observed for HCMV infection in infants in many respects (17–19), and several GPCMV proteins, including the gB and pp65 (GP83) homologs (18, 20), have been shown to provide some protection against congenital GPCMV transmission. However, the combination of gB and pp65 (GP83) homologs in the model demonstrated interference with the anti-gB response compared to that against gB antigen alone following administration of modified vaccinia virus Ankara (MVA)-vectored vaccines (21).

In our studies, we employed a replication-incompetent, single-round infectious vector system based on the prototype Arenavirus lymphocytic choriomeningitis virus (LCMV) clone 13 (22, 23). This expression technology does not elicit vector-neutralizing antibody responses, allowing for administration of homologous booster vaccinations. We describe the construction and in vitro and in vivo characterization of this CMV vaccine candidate expressing HCMV pp65 and gB. These constructs were tested in mice and rabbits and induced significantly higher neutralizing antibody responses than adjuvanted gB protein. Moreover, responses exceeded the titer of human convalescent-phase sera, which was used as a benchmark for comparison. Robust antigen-specific T cell responses were also generated against the vaccine antigen pp65. Finally, the efficacy of both the bivalent and respective monovalent LCMV vectored vaccines was assessed for protection against congenital infection in the GPCMV model.

RESULTS

Design and generation of rLCMV vaccine vectors. A cDNA rescue system was used to generate replication-incompetent rLCMV vaccine vectors (23), where the LCMV surface glycoprotein was precisely replaced by HCMV or guinea pig CMV vaccine antigens (Fig. 1A). The HCMV cytoplasmic tail-deleted gB [gB(dCt)] construct encodes the full ectodomain of HCMV gB, including the membrane anchor (24), followed by an extra arginine residue at position 773 (Fig. 1B). Full-length gB is targeted to apical membranes of polarized cells, and an acidic cluster of amino acids located on the cytosolic part of the protein serves as a signal for gB reinternalization (25, 26). The gB design of rLCMV-HgB(dCt) is aimed to transport gB to the cell surface but to prevent protein reinternalization, which could reduce immunogenicity. A second vector construct was generated to express the full-length sequence of HCMV UL83, encoding the tegument protein pp65. Nuclear localization of pp65 protein after infection was verified using immunofluorescence microscopy (data not shown). Analogous constructs were designed to express proteins derived from guinea pig CMV and were designated rLCMV-GPp65 or rLCMV-GPpp65, respectively.

Characterization of rLCMV vaccine vectors. Owing to LCMV’s complete dependency on the LCMV glycoprotein for cell entry and thereby for virus propagation, the
glycoprotein (GP) (Fig. 1A) gene product was supplemented during vaccine vector production (Fig. 1C) by using a complementing cell line stably expressing the glycoprotein in trans (293-GP). To examine the replication competence of vaccine vectors in the production cell line and, conversely, to demonstrate the inability of the vectors to spread in normal cells, 293-GP and HEK293 cells (293F) were infected with rLCMV-gB(dCt), rLCMV-pp65, or wild-type LCMV virus (LCMV wt) as a control. Both vectors grew efficiently in production cells, reaching titers comparable to that with the wild-type virus (Fig. 2A), whereas no infectious particles were formed in noncomplementing cell lines.
293F cells infected with the vector rLCMV-gB(dCt) (Fig. 2B). This finding verified that rLCMV vector particles that formed in normal cells were glycoprotein deficient and replication incompetent, whereas glycoprotein trans-complementation in production cells provided for efficient particle assembly of vectors.

To confirm expression of the vaccine antigens by rLCMV vectors, lysates of 293-GP cells infected with rLCMV vectors expressing gB(dCt) or pp65 were analyzed by Western blotting (Fig. 2C). rLCMV vector expressing green fluorescent protein (GFP) was used as a control. Native uncleaved gB migrates in the 150-kDa range. During export of the protein to the cell surface, the gB precursor is cleaved by furin, and a surface component with an estimated molecular mass of 116 kDa is formed that is linked by disulfide...
bonds to a transmembrane component with an estimated molecular mass of 55 kDa (27). Vector-expressed gB(dCt) appeared as a single band in the 125-kDa range corresponding to the uncleaved gB(dCt) precursor, whereas the C-terminal cleavage product was detected in the 40-kDa and 25-kDa ranges. A predicted band in the ~65-kDa range was detected for the pp65-expressing vector (28) but not in the GFP-expressing control (Fig. 2C). Membranes were also probed with an antibody specific for LCMV NP protein, which yielded bands of expected size and similar intensity for all vectors tested, confirming that comparable amounts of LCMV vector-expressed protein products were loaded on gels (Fig. 2C). This analysis confirmed that the vaccine vectors expressed the gB(dCt) and pp65 vaccine antigens in infected cells.

**Immune response following rLCMV vector vaccination.** Mice were immunized in a three-dose regimen (days 0, 21, and 105) using 10^5 focus-forming units (FFU) of rLCMV-gB(dCt) or 5 μg recombinant gB protein adjuvanted with EM022, which provided a comparator for adjuvant emulsions with a composition that is comparable to that of MF59 (29). The induction of gB-specific antibody responses was analyzed in an enzyme-linked immunosorbent assay (ELISA) (Fig. 3A, left panel). Immunization stimulated potent gB-specific antibody responses. Responses peaked at day 42 with geometric mean titers (GMT) of 235 μg/ml and 272 μg/ml serum of gB-specific IgG antibodies for rLCMV-gB(dCt) and adjuvanted gB, respectively. Antibody levels were stable during the 9 weeks between the first and second booster. Antibody responses were boosted with a third dose, which increased gB-specific IgG levels from 94 μg/ml to 172 μg/ml serum for LCMV-gB(dCt) and 128 μg/ml to 290 μg/ml serum for adjuvanted gB.

Mouse serum samples obtained at day 42 were chosen for determination of HCMV neutralizing antibody titers. These studies demonstrated a significantly higher neutralization capacity of sera induced by rLCMV-gB(dCt) than by adjuvanted recombinant gB protein and a slightly higher neutralization capacity than the mean response of human convalescent-phase sera, used as a benchmark for this analysis (Fig. 3A, right panel).

Neutralizing antibody titers directed against gB are significantly augmented by adding complement in HCMV neutralization assays (30). This prompted us to analyze the subclass of antibodies induced by vaccination, insofar as different subtypes of IgG antibodies differ in their ability to activate complement. ELISA analysis of peak titer mouse serum (day 42) containing similar levels of total gB-specific IgG using IgG subclass-specific secondary antibodies indicated that animals vaccinated with adjuvanted recombinant gB predominantly generated IgG1 antibodies (85%), which only poorly activate complement, whereas the rLCMV vector induced mainly gB-specific IgG2b and IgG2c antibodies (40% and 54%, respectively), which efficiently activate complement (Table 1).

Vaccinated rabbits demonstrated potent and dose-dependent induction of gB-specific antibodies exhibiting HCMV neutralizing capacity in the medium- and high-dose groups (Fig. 3B).

To analyze the immunogenicity of the rLCMV vector expressing the T cell antigen pp65, mice were vaccinated intramuscularly (i.m.) by utilizing 10^5 or 10^3 FFU on days 0 and 28 of the experiment. Vaccination with 10^5 FFU induced robust pp65-specific CD8^+ responses, with a mean magnitude of 1.8% pp65-specific CD8^+ cells after priming and 3.0% after a booster vaccination (Fig. 3C). pp65-specific T cells were also detected in the low-dose group, reaching mean levels of 0.86% pp65-specific CD8^+ cells after priming or 1.0% after boost, respectively.

**Interference in bivalent vector composition.** Observations suggesting interference between gB and pp65 antigens in previous studies (21, 31) prompted us to analyze if any interference was observed when rLCMV-gB(dCt) and rLCMV-pp65 vaccines were coadministered and compared to monovalent immunization. Immunogenicity of monovalent [rLCMV-gB(dCt) or rLCMV-pp65] and bivalent [rLCMV-gB(dCt) plus rLCMV-pp65 mixed in a 1:1 ratio] vaccines was analyzed after i.m. vaccination of C57BL/6 mice with a dose of 10^5 FFU per vector on days 0 and 28. gB-specific IgG ELISA
FIG 3  Immune analyses of rLCMV-gB and pp65 vectors. Error bars represent 95% confidence intervals for serological assays and standard deviations for T cell analyses. (A) Immune in C57BL/6 mice. A total of 1 × 10^7.

(Continued on next page)
analysis was conducted using sera obtained on day 49 and indicated no significant difference in the magnitude of gB-specific antibody induction in the monovalent versus bivalent vaccination (Fig. 3D, left panel). Analogously, determination of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), or interleukin-2 (IL-2)-producing pp65-specific CD8+ responses indicated no significant difference between the monovalent versus bivalent vaccines (Fig. 3D, right panel), confirming that there was no interference when vaccine was administered as a bivalent combination vaccine.

Immunogenicity and protective efficacy of rLCMV vectors expressing gB(dCt) and pp65 from guinea pig CMV. Antibody responses measured by ELISA in guinea pig dams vaccinated with constructs expressing GPCMV antigens homologous to the HCMV-specific antigens demonstrated excellent immunogenicity with strong and uniform (narrow 95% confidence interval [CI]) antibody responses to GPgB(dCt) in both the monovalent and bivalent vaccines, in agreement with results from mouse studies (Fig. 4A). The rLCMV-GPgB(dCt) vaccine elicited robust neutralizing antibody titers alone or when administered in combination with rLCMV-GPpp65, indicating no interference between antigens (21) (Fig. 4B) (in both cases, P < 0.0001 compared to the irrelevant vector control). Indeed, animals receiving the bivalent combination of rLCMV-GPgB(dCt) and rLCMV-GPpp65 had a significantly (P = 0.0006) stronger neutralizing response than animals vaccinated with rLCMV-GPgB(dCt). Consistent with the ELISA data, sera from animals immunized with rLCMV-GPgB(dCt) were significantly more strongly neutralizing than sera from animals immunized with adjuvanted GPgB protein (P < 0.0001).

Splenocytes from animals immunized with rLCMV-GPpp65 showed stronger reactivity in enzyme-linked immunosorbent spot (ELISPOT) assays than cells from control animals receiving a vector expressing an irrelevant protein (rLCMV-GFP) (Fig. 4C). There was not a significant difference in reactivity between splenocytes from animals immunized with monovalent rLCMV-GPpp65 and animals immunized with the bivalent vaccine. Peptide mapping of GP83 epitopes following rLCMV-GPpp65 vaccination was comparable to that observed during natural GPCMV infection (data not shown). Responses to two previously identified immunodominant peptides (LGIHVFFDN and CQEQVFVKS) were observed following vaccination (32).

Reduction of viral load following rLCMV-vectored vaccination. Viral loads in all vaccinated groups were significantly lower at day 7 compared to the rLCMV-GFP control response. The viral load after rLCMV-GPgB(dCt) vaccination was (1.6 ± 0.3) × 10⁶ copies/ml (mean ± standard deviation), compared to (1.7 ± 0.6) × 10⁶ copies/ml

TABLE 1 Results of IgG subclass analysis

| Vaccine       | Mean % of IgG subclass a |
|---------------|-------------------------|
|               | IgG1 | IgG2b | IgG2c | IgG3 |
| recgB + FA    | 85   | 11    | 3.8   | 0.1  |
| rLCMV-gB(dCt)| 4.0  | 40    | 54    | 1.5  |

*The mean percentages were calculated from subclass titers for individual animals, e.g., % IgG1 = (IgG1/(IgG1 + IgG2b + IgG2c + IgG3)) × 100.

FIG 3 Legend (Continued)

10⁶ FFU per dose of rLCMV or 5 μg per dose of adjuvanted recombinant gB were administered i.m. on days 0 and 21. (Left) Antibody induction and persistence were measured by ELISA. (Right) Levels of neutralizing antibodies in sera collected on day 42 after two administrations of rLCMV-gB(dCt) or adjuvanted recombinant gB, in comparison to 5 human sera from CMV-infected subjects which covered a typical range of titers for naturally induced humoral immunity. (B) Immunogenicity in New Zealand White rabbits; 1 × 10⁵ FFU, 1 × 10⁴ FFU, or 1 × 10³ FFU of rLCMV-gB(dCt) was administered i.m. on days 0, 28, and 56. (Left) Antibody induction and persistence measured by ELISA. (Right) Levels of neutralizing antibodies in sera collected on day 69 after three administrations of rLCMV-gB(dCt). (C) T cell responses induced by pp65 vector in C57BL/6 mice; a dose of 1 × 10⁵ or 1 × 10⁴ FFU was administered i.m. on days 0 and 28. T cell analysis of splenocytes was performed at day 10 (d10 postprime) and day 38 (day 10 postboost) by ICS. Frequencies of pp65-specific CD8 T cells expressing at least one of the cytokines IFN-γ, TNF-α, or IL-2 are shown. (D) Immunogenicity of monovalent and bivalent rLCMV formulations in C57BL/6 mice; 1 × 10⁵ FFU of rLCMV vector were administered i.m. on days 0 and 28; gB-specific antibody induction and pp65-specific CD8 T cell responses were measured at day 49 by ELISA and ICS.
Immunogenicity analyses of rLCMV GPCMV vectors in guinea pigs. Error bars represent 95% confidence intervals. A total of \(8 \times 10^5\) FFU per dose of each rLCMV vector were administered i.m., or 50 µg per dose of adjuvanted recombinant GpG B was administered s.c. on days 0, 30, and 60. Animals were challenged with \(1 \times 10^5\) PFU GPCMV after day 155. (A) Antibody induction measured by ELISA. (B) Neutralizing titers in sera at day 103. The titers in individual sera are shown with geometric means and 95% CI indicated. Sera without neutralizing effects at the lowest dilution were assigned a titer of half the limit of detection (LOD). Hyperimmune serum was derived from an animal immunized with adjuvanted recombinant gB protein (50 µg in IFA) and subsequently infected with GPCMV. Statistical comparisons were made by ANOVA on log-transformed values.
in the rLCMV-GPpp65 group, $(5.8 \pm 3.1) \times 10^5$ copies/ml in the group that received bivalent recombinant baculovirus-expressed GpB (truncated at Pro92) admixed with incomplete Freund’s adjuvant (recGpB plus FA), and $(4.5 \pm 1.0) \times 10^6$ copies/ml in the rLCMV GFP controls (Fig. 5A). Administration of the bivalent vaccine rLCMV-gB(dCt)/GPpp65 demonstrated a significant reduction of viral load compared with rLCMV gB(dCt) vaccine administered alone ($P = 0.0386$) (Fig. 5A). The analysis of reduction in viral load after the bivalent vaccine, compared with rLCMV GPpp65 vaccine alone, did not reach statistical significance ($P = 0.07$). However, the viral load of $(1.65 \pm 0.3) \times 10^6$ copies/ml when the single-antigen vaccine groups [rLCMV-GPpgB(dCt) and rLCMV-GPpp65] were combined was significantly higher than that of the bivalent rLCMV-GPgB(dCt)/GPpp65 combination group ($P = 0.03$, t test) (Fig. 5B).

Protection against mortality and disease following rLCMV-vectored vaccination. Preconception vaccination resulted in a significant decrease in pup mortality after challenge (Table 2). The mortality rate was 13% in the rLCMV-GPgB(dCt) group, 38% with rLCMV-GPpp65, 8% with bivalent rLCMV-GPpgB(dCt)/GPpp65, 20% with recGpB/Freund’s adjuvant, and 93% in the rLCMV-GFP control group. Furthermore, the mortality rate in pups born to dams vaccinated with the single antigen rLCMV-GPpp65 was significantly higher than for those born to the rLCMV-GPgB(dCt)-vaccinated group ($P = 0.03$, t test).
0.0059) and for those born to the bivalent rLCMV-GPpgB(dCt)/GPpp65-vaccinated group (P = 0.0024, Fisher’s exact test). There were no significant differences in mortality between the rLCMV-GPpgB(dCt) (13%) and rLCMV-GPpgB(dCt)/GPpp65 (8%) groups. Pup weights were also significantly higher in the vaccinated versus rLCMV-GFP control groups (Fig. 6).

Duration of pregnancy following SG-GPCMV challenge in the control group was 13.4 ± 2.6 days, which was significantly lower (P < 0.01, Kruskal-Wallis) than in all of the vaccine groups [23.9 ± 2.4 in the rLCMV-GPpgB(dCt) group; 25.3 ± 4 in the rLCMV-GPpp65 group; 33.9 ± 6.4 in the bivalent rLCMV-GPpgB(dCt)/GPpp65 group; 35.1 ± 4.4 in the recGPgB plus FA group]. In the rLCMV-GPpgB(dCt) vaccine group, 41/55 pups (75%), compared to 22/33 (67%) in the rLCMV-GPpp65 group, had congenital GPCMV infection, as evidenced by positive PCR detection in pup lung, liver, or spleen. Congenital infection was observed in 22/40 (55%) of the recGPgB plus FA group, and in 20/40 (50%) of pups in the control group. In the bivalent rLCMV-GPpgB(dCt)/GPpp65 vaccine group, congenital GPCMV infection was observed in 18/38 pups (53%). One pup from the control group and six pups from the rLCMV-GPpp65 group were unavailable for PCR testing. When the bivalent rLCMV-GPpgB(dCt)/GPpp65 group was compared to both monovalent LCMV vector groups combined, the rate of congenital transmission was significantly reduced (53% compared to 72%; P < 0.05, Fisher’s exact test).

**DISCUSSION**

Vaccines against HCMV-associated disease targeting both congenital infection (33) and HCMV-associated disease in patients who have received solid organ or hematopoietic stem cell transplant are needed (34, 35). All vectored and subunit vaccines

| Vaccine group                        | Total no. of litters | No. of live pups | No. of dead pups | % mortality |
|--------------------------------------|----------------------|------------------|------------------|-------------|
| rLCMV-GPpgB(dCt)                    | 17                   | 48               | 7                | 13*         |
| rLCMV-GPpp65                        | 10                   | 24               | 15               | 38*         |
| rLCMV-GPpgB(dCt) + GPpp65           | 12                   | 35               | 3                | 8*/f        |
| recGPgB + FA                        | 13                   | 32               | 8                | 20*         |
| rLCMV-GFP                           | 11                   | 3                | 38               | 93          |

* P < 0.0001 versus rLCMV-GFP; f, P < 0.01 versus rLCMV-GPpgB(dCt).

**FIG 6** Improved pup weights and reduced pup mortality conferred by preconception vaccination. Pup weights were significantly improved in all vaccine groups compared to the rLCMV-GFP control group (P < 0.0001, Kruskal-Wallis and Dunn’s multiple comparison tests). Open circles, live-born pups; closed circles, stillborn pups.
evaluated in clinical trials to date have included the immunodominant gB glycoprotein, and several have included the dominant T cell target pp65 (ppUL83), with or without the major IE1 gene product (4–7). However, it is not known whether inclusion of T cell targets improves protective efficacy compared to gB administered alone. Moreover, one study using a recombinant MVA system in a guinea pig model suggests that inclusion of the pp65 homolog in a two-component vaccine decreased the immunogenicity of gB, compared to an MVA-vectored gB vaccine administered alone (21). In the present study, we demonstrated that immunization with both HCMV and GPCMV gB and pp65 rLCMV vectors did not show interference; moreover, there were significant additive benefits of both gB and pp65, both in reducing the magnitude of maternal viremia following viral challenge and in improving pregnancy and pup outcomes.

The vaccines described in this report are based on a recombinant clone 13 strain of LCMV that exhibits a natural tropism for dendritic cells and elicits both robust CD8+ T cell and antibody responses (23). Vectors were generated by replacing the essential GP gene with the HCMV or GPCMV antigens of interest. Previous work with rLCMV-vectored vaccines elicited responses that were equivalent or superior to those elicited by recombinant adenovirus 5 or recombinant vaccinia virus, both in magnitude of the cytotoxic T lymphocyte response and cytokine profiles. These rLCMV vaccines were also more protective in several models, including a Listeria monocytogenes challenge model (23). Importantly, in contrast to recombinant adenovirus 5, rLCMV has been previously shown not to elicit any vector-specific antibody immunity, allowing for the potential of readministration for booster vaccination (23). This would be of particular relevance for a maternal HCMV vaccine, for which periodic booster immunizations of women of child-bearing age may be required to confer protection against reinfection and subsequent congenital transmission (36) throughout their childbearing years.

rLCMV vectors expressing the HCMV antigens gB(dCt) and pp65 elicited robust, antigen-specific humoral and cellular immune responses in mice and rabbits in a dose-dependent manner, and the gB-binding antibody response was comparable in magnitude and durability to that following adjuvanted subunit gB protein. However, the HCMV-neutralizing titers of mice immunized with rLCMV-gB(dCt) exceeded responses of mice immunized with adjuvanted gB.

In contrast to a previous report by Cardin (37), the current study demonstrated that gB-containing LCMV vaccines were superior to GPpp65 LCMV vaccines in protecting against pup mortality and in reducing pup viral load. Cardin et al. observed 23% pup mortality in the GPgB-vaccinated group and 26% in the pp65-vaccinated group, compared to 49% mortality in control pups (37). In the present study, we observed 93% pup mortality in the control group, which was reduced to 39% in the GPpp65-vaccinated and 13% in the GPgB-vaccinated groups. One reason for these differences might be the improved immunogenicity of the GPgB vaccine, since the GPCMV HK1-GPgB(dTM) LCMV vector construct used in the Cardin study encoded a transmembrane-deleted protein comprising the entire extracellular domain of GPCMV gB (through Arg685) and the entire intracellular domain (amino acids [aa] 762 to 901). In contrast, the dCt construct in the current study was a C-terminally truncated gB consisting of the N-terminal 758 amino acids of gB (encoding the predicted full ectodomain of gB) and the membrane anchor that transports gB to the cell surface and prevents protein reinternalization via deletion of cytosolic internalization signals (24–26). Although we propose that the enhanced immunogenicity of gB in the dCt construct was responsible for the improved pregnancy outcomes observed compared to those with the monovalent rLCMV-GPpp65 vaccine, the ELISA and neutralization assays were performed using different methodologies, making direct comparisons of the serologic results between the two studies problematic.

Notably, immunization with bivalent rLCMV-GPgB(dCt) plus rLCMV-GPpp65 resulted in a statistically significant reduction of maternal DNAemia and reduced pup mortality, compared to monovalent vaccination with either vaccine alone. Although there have been no side-by-side comparisons of single-antigen HCMV gB vaccines with multiantigen gB and pp65 vaccines in clinical trials using acquisition of HCMV infection as a
study endpoint, it is noteworthy that monovalent adjuvanted recombinant gB vaccine has demonstrated only modest efficacy, in the range of 40 to 50%, in clinical trials of adolescents and young women (9, 10). Since a major goal of vaccination is to confer protection against HCMV transmission in infants born to young women of childbearing age, the additive benefit of combining both gB and pp65 in the guinea pig model may be relevant to evaluation of future bivalent HCMV vaccines in clinical trials.

Our study results are in marked contrast to those from a prior study of MVA-vectored gB and pp65 vaccines, where it was observed that the addition of pp65 to a gB subunit vaccine not only interfered with antibody responses but also correlated with diminished protection against congenital GPCMV infection, compared to gB vaccine given alone (21). Interestingly, in our study, the neutralizing response was significantly higher in animals administered the bivalent vector vaccine than in animals who received only rLCMV-GBgB(dCt). This may have been due to the higher total vector dose in the bivalent vaccine group contributing to the stronger overall immune response, although these differences were not observed in the ELISA.

In conclusion, these data demonstrate that both vectors, LCMV-gB(dCt) and LCMV-pp65, are highly immunogenic for both HCMV and GPCMV antigens. Immunization with the HCMV vaccine constructs induces robust CMV gB-specific neutralizing antibody responses and robust pp65-specific IFN-producing CD8+ responses in mice. Similarly, an LCMV-vectored vaccine based on the GPCMV gB homolog induces high-titer ELISA and neutralizing antibody responses, with superior immunogenicity compared to gB with Freund’s adjuvant, in guinea pigs. The GPCMV rLCMV-GPpp65 vaccine construct was demonstrated to induce T cell responses, as measured in an IFN-γ ELISPOT assay, in guinea pigs. Finally, this study demonstrated an additive benefit of combining both a neutralizing antibody target (gB) and a T cell target (GP83 [pp65 homolog]) in a bivalent vaccine strategy targeting prevention of maternal and fetal disease in the GPCMV model, and our findings support further testing of rLCMV-based HCMV vaccines in clinical trials.

MATERIALS AND METHODS

Guinea pigs. Outbred Hartley guinea pigs, confirmed to be GPCMV seronegative by ELISA (18), were purchased from Elm Hill Laboratories (Chelmsford, MA) and housed under university-approved conditions.

Generation of LCMV-vectored HCMV and GPCMV vaccine constructs. rLCMV vectors were generated and titrated as described previously (22, 23). Briefly, the coding sequence (cDNA) of individual vaccine antigens was synthesized by Genscript (USA) and inserted into a plasmid harboring a GP-deleted S segment of LCMV clone 13 under the control of a murine polymerase I (pol I) promoter. rLCMV-gB(dCt) encodes the ectodomain and transmembrane domain of HCMV UL55 (gB; GenBank accession number AY446894) derived from strain Merlin (amino acids 1 to 772), followed by an additional arginine residue at position 773 to aid in membrane anchoring. A corresponding vector construct was based on guinea pig CMV gB derived from strain 22122 (GenBank accession number KCS03762) and designated rLCMV-GPgpB(dCt), encoding the N-terminal 758 amino acids of the protein (Fig. 1B). Analogously, vectors encoding the entire UL83/GP83 (pp65) protein of HCMV strain AD169 or GPCMV strain 22122 were generated and designated rLCMV-pp65 or rLCMV-GPpp65, respectively. Consensus sequencing confirmed transgene sequences before recovering rLCMV vectors by using a pol I/pol II rescue system (22).

Vector stocks were generated in suspension in HEK293 production cells (293-GP) genetically engineered to express the LCMV glycoprotein. Cells were seeded in shake flasks at 3 × 10^6 cells/ml in 30 ml CDMA4HER293 medium (GE Healthcare) supplemented with 4 mM stable glutamine and 100 μg/ml Geneticin and infected with rescued vector at a multiplicity of infection (MOI) of 0.001. At day 3 postinfection, supernatants were cleared from cells and debris by low-speed centrifugation (500 g, 5 min, 2 °C), aliquoted, and frozen below −60°C. Vector stocks were titrated using an LCMV NP-specific focus-forming unit (FFU) assay as previously described (37). The generation and purification of recombinant GPCMV gB expressed in baculovirus have been described previously (18).

Growth kinetics of vaccine vectors. 293-GP or noncomplementing HEK293F cells (Invitrogen) were inoculated with vector stocks generated as described above. Aliquots from individual cultures were drawn at 2, 24, 48, 72, and 96 h postinfection. Supernatant was cleared by low-speed centrifugation, aliquoted, and frozen below −60°C. Individual samples were titrated based on the FFU. The wild-type LCMV virus (LCMV-wt) used as a control in the growth curve analyses is a recombinant virus which expresses an identical LCMV glycoprotein antigen sequence as the production cell line 293-GP.

Protein expression in vector-infected cells. HEK293 suspension cells constitutively expressing LCMV glycoprotein (293-GP) were infected with vectors at an MOI of 0.001 and incubated for 72 h. Whole-cell lysates were resolved by 4-to-12% gradient SDS-PAGE (NuPAGE 4-to-12% bis-Tris gel; Novex) and transferred to nitrocellulose membranes (Novex) using iBlot (Invitrogen). Nonspecific binding sites were blocked in 3% nonfat dry milk in Tris-buffered saline Tween 20 (TBST) 1% at 4°C for 1 h. Membranes were incubated in primary antibodies in 5% nonfat dry milk in TBST at 4°C overnight. Membranes were washed three times in TBST and incubated in horseradish peroxidase-conjugated secondary antibodies in 5% nonfat dry milk in TBST at room temperature for 1 h. Membranes were washed three times in TBST and developing on peroxidase substrate solution (Bio-Rad). Blots were imaged with a ChemiDoc XRS+ gel documentation system (Bio-Rad).
were blocked by incubation with 5% blotting-grade blocker (catalog number 170-6404; Bio-Rad Laboratories) in Tris-buffered saline with 0.1% Tween 20 (1× TBST). Murine monoclonal anti-HCMV gB IgG antibody (diluted 1:750; Sino Biological), which binds the C-terminal part of the gB ectodomain, murine monoclonal anti-HCMV pp65 antibody (diluted 1:200; Abcam), or rabbit anti-LCMV sera (diluted 1:2,000; Doron Merkler, Faculty of Medicine, Department of Pathology and Immunology, University of Geneva) were used for Western blot analyses. Secondary antibodies included horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch) or donkey anti-rabbit IgG antibody (Jackson Immunoresearch), respectively. Enhanced chemiluminescence staining was visualized using an Amersham chemiluminescence reader (Amersham/GE Life Sciences).

Immunization of mice and rabbits. Groups of 6-week-old female C57BL/6 mice (n = 5 or 10 animals), obtained from Charles River Laboratories (Chatillon-sur-Chalaronne, France), were immunized at day 0 and boosted on day 21 or day 28 (in one experiment animals were boosted again on day 105) by i.m. injection of 50 µl of vaccine candidate in both hind legs (total volume, 100 µl). LCMV vectors were prepared in diluent (25 mM HEPES, 150 mM NaCl, 0.01% Pluronic F68; pH 7.4) with a final concentration of 10% sorbitol and frozen below −60°C until use. Control animals were immunized with a 1:1 mixture of EM022 adjuvant (Infectious Disease Research Institute, Seattle, WA), and 5 µg recombinant gB protein (Sino Biological) comprising the extracellular domain (Met-1 to Lys-700) linked with the cytoplasmic domain (Arg-777 to Val-907) of the gB protein from human CMV strain Towne. Serum samples and/or spleens were harvested on the specified days. Mouse experiments were performed at Preclin Biosystems AG (Epalinges, Switzerland).

Groups of five 13- to 15-week-old female New Zealand White rabbits, sourced from Charles River Laboratories (Chatillon-sur-Chalaronne, France), were immunized on day 0 and boosted on days 28 and 56 by i.m. injection of 250 µl of vaccine candidate at 4 sites (total volume, 1,000 µl). Groups of 6-week-old female C57BL/6 mice (n = 5 or 10 animals), obtained from Charles River Laboratories (Chatillon-sur-Chalaronne, France), were immunized at day 0 and boosted on day 21 or day 28 (in one experiment animals were boosted again on day 105) by i.m. injection of 50 µl of vaccine candidate in both hind legs (total volume, 100 µl). LCMV vectors were prepared in diluent (25 mM HEPES, 150 mM NaCl, 0.01% Pluronic F68; pH 7.4) with a final concentration of 10% sorbitol and frozen below −60°C until use. Control animals were immunized with a 1:1 mixture of EM022 adjuvant (Infectious Disease Research Institute, Seattle, WA), and 5 µg recombinant gB protein (Sino Biological) comprising the extracellular domain (Met-1 to Lys-700) linked with the cytoplasmic domain (Arg-777 to Val-907) of the gB protein from human CMV strain Towne. Serum samples and/or spleens were harvested on the specified days. Mouse experiments were performed at Preclin Biosystems AG (Epalinges, Switzerland).

Mouse IgG subclass-specific anti-HCMV ELISA. Antibody responses to gB of HCMV were measured by indirect ELISA using recombinant gB (Sino Biological) as coating antigen on 96-well microplates (0.5 µg/ml, 100 µl per well, overnight at 4°C) and a goat HRP-conjugated mouse-antibody IgG polyclonal secondary antibody (Jackson Immunoresearch). Serial dilutions of a mouse monoclonal gB-specific IgG1 antibody (Sino Biological) served as reference standard, with results expressed as micrograms per milliliter monoclonal antibody (MAb) equivalent concentration. Titers were log transformed prior to statistical analysis.

Rabbit IgG anti-HCMV gB ELISA. Antibody responses were determined analogously to the mouse anti-HCMV ELISA, using a purchased rabbit anti-HCMV gB-specific polyclonal antibody (Bioss) as a reference standard and a goat anti-rabbit secondary antibody (Jackson Immunoresearch). Results were expressed as rabbit ELISA units (rEU) per milliliter.

Guinea pig anti-GCMV gB ELISA. Titers for guinea pig anti-GCMV were determined by ELISA using recombinant His-tagged GpGB protein produced in CHO cells as target antigen (0.05 µg/ml; Evitria) in nickel-coated plates (Qiagen). Analogous to the mouse anti-HCMV ELISA, the guinea pig ELISA titers were defined based on a pool of standard hyperimmune guinea pig serum with an assigned endpoint titer as reference; results were expressed as guinea pig ELISA units (gpEU) per milliliter.

GPCMV-specific neutralization assays. The GFP-tagged recombinant vJZ848 virus was used for neutralization assays as previously reported (21). Neutralization assays were performed on guinea pig fibroblast lung cells (ATCC CCL 158). Neutralizing titers were defined as the dilutions resulting in a reduction of ≥50% in the total number of GFP-positive foci. A hyperimmune serum from a guinea pig immunized with adjuvanted gB and subsequently infected with GPCMV was used as a positive control.

HCVM-specific neutralization assays. Virus neutralization assays were performed at Virginia Commonwealth University in the laboratory of M. McVoy (38–41) (Fig. 3A) and by the authors by applying an analogous method (Fig. 3B). The virus neutralization procedure was based on human ARPE-19 retinal pigment epithelial cells (ATCC CRL-2302) and a GFP-expressing recombinant HCMV virus based on strain Towne (TS15-rN [38]). Threefold serial dilutions of sera (50 µl) in medium were prepared in duplicate in 96-well plates and mixed with 50 µl (300 PFU) TS15-rN in medium containing 10% guinea pig serum (as a source of complement; Merck-Millipore) per well. After incubation at 37°C for 1 h, 50-µl aliquots were transferred to black-walled clear, flat-bottomed half-area 96-well plates (Corning) that contained sub-confluent ARPE-19 cells in 50 µl medium (MOI, 0.02). The number of fluorescent cells was measured 4 days postinfection (using a Bioreader 6000 plate reader; Bio-Sys). A 4-parameter sigmoidal curve was fitted through the data points with the neutralizing titer calculated using GraphPad Prism, version 6.04, and expressed as the reciprocal of the serum dilution that reduced the number of infected cells by 50% compared to the medium control (NT-50).

HCVM pp65-specific T cell analyses. A flow cytometry-based intracellular cytokine staining (ICS) assay was used to determine the frequencies of pp65-specific CD8 T cells expressing IFN-γ, TNF-α, or IL-2. Splenocytes from study animals (2 × 10⁶ cells) were incubated with a pool of 17 H-2b-restricted pp65-immunoreactive peptides (42) at a concentration of 1 µg/ml per peptide or with medium alone...
A dose of 1 administration (described below). Following vaccination, animals were mated, pregnancy was established, and fetal viremia assessment. Mann-Whitney analysis was employed to compare the rLCMV-GPgpB(dCt) versus the rLCMV-GPpp65 group. Pooled monovalent groups were compared to the rLCMV-GPgpB(dCt)/GPpp65 bivalent group by using the rLCMV-GPpp65 group, the bivalent rLCMV-GPgpB(dCt) plus rLCMV-GPpp65 group, and the rLCMV-GFP group were sacrificed for ELISPOT analyses 7 days following the second (n = 3) or third (n = 3) administration (day 3). Vaccination, animals were mated, pregnancy was established, and dams were challenged with salivary gland-adapted GPCMV (SG-GPCMV) at a dose of 1 × 10^6 PFU by the s.c. route (43). Pregnancy outcomes were then monitored.

**ELISPOT assay.** Mouse monoclonal anti-IFN-γ antibodies (N-G3 and V-E4) were a gift from Hubert Schäfer (21, 44). ELISPOT assays were carried out as previously described, with minor modifications (32). Peptides spanned the coding sequence of GP83 in 9-aa-long fragments with 5-aa overlaps (140 total peptides). Spleens were harvested from guinea pigs at 28 to 32 days following the third vaccination, and purified splenocytes (1 × 10^6) were mixed with 50 μg/ml stimulant (peptide pools) or controls (no-stimulation, dimethyl sulfoxide control, or positive control concanavalin A at 20 μg/ml). Secondary antibody and developing reagents were added as previously described (32), and spots were counted with an AID Elispot reader system using Elispot 6.0-iSpot (Autoimmune Diagnostika GmbH, Straßberg, Germany).

**Real-time qPCR analysis.** Maternal blood obtained on day 7 post-challenge with SG-GPCMV was analyzed for viral load via quantitative PCR (qPCR) (43), with the results expressed as the number of genome copies per milliliter of blood. Since the limit of detection was approximately 200 copies/ml, a level of 100 copies/ml was assigned to negative samples. Organs (liver, lung, and spleen) from stillborn pups or live-born pups sacrificed within 72 h of delivery were homogenized and DNA was extracted for qPCR to evaluate congenital GPCMV transmission as previously described (21).

**Statistical analyses.** GraphPad Prism (version 6.0) was used for statistical analyses. Pup mortality and transmission rates were compared using Fisher’s exact test with two-sided comparisons. Pup weights in pregnancy/challenge studies and pregnancy duration were compared using the Kruskal-Wallis test followed by Dunn’s multiple-comparison test. Guinea pig serum neutralizing titers and ELISA titers were compared using Student’s t test on log-transformed data. DCD8+ T cell responses were compared with Student’s t test. One-way ANOVA followed by Tukey’s multiple-comparison test was used for comparing each group to the control for day 7 maternal viremia assessments. Mann-Whitney analysis was employed to compare the rLCMV-GPgpB(dCt) versus the rLCMV-GPgpB(dCt)/GPpp65 group. Pooled monovalent groups were compared to the rLCMV-GPgpB(dCt)/GPpp65 bivalent group by using the t test.

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**REFERENCES.**

1. Griffiths P, Baraniak I, Reeves M. 2015. The pathogenesis of human cytomegalovirus. J Pathol 235:288–297. https://doi.org/10.1002/path.4437.

2. Boechk M, Geballe AP. 2011. Cytomegalovirus: pathogen, paradigm, and puzzle. J Clin Invest 121:1673–1680. https://doi.org/10.1172/JCI45449.

3. Arvin AM, Fast P, Myers M, Plotkin S, Rabinovich R. 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.

4. Khanna R, Diamond DJ. 2006. Human cytomegalovirus vaccine: time to look for alternative options. Trends Mol Med 12:26–33. https://doi.org/10.1016/j.molmed.2005.11.006.

5. Sung H, Schleiss MR. 2010. Update on the current status of cytomegalo-
lovi virus vaccines. Expert Rev Vaccines 9:1303–1314. https://doi.org/10.1586/er.10.125.

6. Fu TM, An Z, Wang D. 2014. Progress on pursuit of human cytomegalo-
lovirus vaccines for prevention of congenital infection and disease. Vaccine 32:2525–2533. https://doi.org/10.1016/j.vaccine.2014.03.057.

7. McVoy MA. 2013. Cytomegalovirus vaccines. Clin Infect Dis 57(Suppl 4):S196–S199. https://doi.org/10.1093/cid/cits87.

8. Sylweste AH, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleetl PR, Grise CW, McKeen NA, Kern F, Nelson JA, Picker LJ. 2005. Broadly
healthy human cytomegalovirus-specific CD4 + and CD8 + T cells domi-
inate the memory compartments of exposed subjects. J Exp Med 202:
673–685. https://doi.org/10.1084/jem.20050882.

9. 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.

10. Bernstein DJ, Munoz FM, Callahan ST, Rupp R, Wootton SH, Edwards KM, Turley CB, Stanberry LR, Patel SM, McNeal MM, Pichon S, Amegahesi 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.

11. Griffiths PD, Stanton A, McCarroll E, Smith C, Osman M, Harber M, Davoust A, Slocombe AT, Wheeler D, O’Beirne J, Thorburn D, Paté D, Atkinson CE, Pichon S, Sweny P, Lanzman M, Woodford E, Rothwell E, Old N, Kinyanjui R, Haque T, Atabani S, Luck S, Prideaux S, Milne RS, Emery VC, Burroughs AK. 2011. Cytomegalovirus glycoprotein-B vaccine with MR95 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial. Lancet 377:1256–1263. https://doi.org/10.1016/S0140-6736(11)60136-0.

12. Khanjan-Dabaja MA, Breeck M, Wilck MB, Langston AA, Chu AH, Wloch MK, Gutierrez DF, Smith LR, Rolland AP, Kenney RT. 2012. A novel
therapeutic cytomegalovirus DNA vaccine in allogeneic haemopoietic stem-cell transplantation: a randomised, double-blind, placebo-
controlled, phase 2 trial. Lancet Infect Dis 12:290–299. https://doi.org/10.1016/S1473-3099(11)70344-9.

13. Gönçzöl E, Berencsi K, Pincus S, Endreza Y, Méric C, Paolietti E, Plotkin SA. 1995. Preclinical evaluation of an ALVAC (canarypox)-human cytome-
glovirus glycoprotein B vaccine candidate. Vaccine 13:1080–1085.

14. Berencsi K, Gyulai Z, Gönçzöl E, Pincus S, Cox WI, Michelson S, Kari L, Kohn J, Johnson S, Kreppel F, Kochanek S, Broek Mv, Radbruch A, Lévy F, Lambert PH, Siegrist CA, Restifo NP, Löhning M, Ochsenbein AF, Kharfan-Dabaja MA, Boeckh M, Gilbert PB. 2016. Search continues for a CMV vaccine for endocarditis from the plasma membrane. J Virol 73:8677–8688.

15. Schleiss MR. 2013. Developing a vaccine against congenital cytomegalo-
virus: new prospects for prevention and therapy. Pediatr Clin North Am 60:
7374–7386. https://doi.org/10.1016/j.pcl.2014.06.019.

16. Ross SA, Arora N, Novak Z, Fowler KB, Britt WJ, Boppana SB. 2010.
Cytomegalovirus reinfactions in healthy serouninfected women. J Infect Dis 201:386–389. https://doi.org/10.1086/649902.

17. Swanson EC, Gillis P, Hernandez-Alvarado N, Fernandez-Alarcón C, Schmitt M, Zabel JC, Wussow F, Diamond DJ, Schleiss MR. 2015. Con-
jugation with adenoviral glycoprotein B with a truncated gB(94-265) (GP83) vaccine for congenital cytomegalovirus infection in a guinea pig model: Inclusion of GP83 reduces gB antibody response but both
vaccine approaches provide equivalent protection against pup mortality. Vaccine 33:4013–4018. https://doi.org/10.1016/j.vaccine.2015.06.019.

18. Flatz L, Berghalter A, de la Torre JC, Pichorner DD. 2006. Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA. Proc Natl Acad Sci U S A 103:4663–4668. https://doi.org/10.1073/pnas.0605201103.

19. Flatz L, Hegazy AN, Berghalter A, Verschoor A, Claus C, Fernandez M, Gattinoni L, Johnson S, Kreppel F, Kochanek S, Broek Mv, Radbruch A, Lévy F, Lambert PH, Siegrist CA, Restifo NP, Löhning M, Ochsenbein AF, Nabel GJ, Pichorner DD. 2010. Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8 + T cell immunity. Nat Med 16:339–345. https://doi.org/10.1038/nm.2104.

20. Tugzov S, Maudidi E, Xiao J, Zheng Z, Pereira L. 1998. Human cytomegalovirus glycoprotein B contains autonomous determinants for vectorial targeting to apical membranes of polarized epithelial cells. J Virol 72:
7374–7386.

21. Fox CB, Haensler J. 2013. An update on safety and immunogenicity of vaccines containing emulsion-based adjuvants. Expert Rev Vaccines 12:
103–118. https://doi.org/10.1586/14760584.2013.811188.

22. Novakovic B, Gmeiner A, Samow P, Levine AJ, Fleckenstein B. 1984. Physical
mapping of human cytomegalovirus genes: identification of DNA se-
quences coding for a virion phosphoprotein of 71 kDa and a viral 65-kDa polypeptide. Virology 134:91–102. https://doi.org/10.1016/0042-
6828(84)90275-7.

23. Swanson EC, Schleiss MR. 2013. Congenital cytomegalovirus infection: new prospects for prevention and therapy. Pediatr Clin North Am 60:
335–349. https://doi.org/10.1016/j.pcl.2012.12.008.

24. Boeckh M, Gilbert PB. 2016. Search continues for a CMV vaccine for transplant recipients. Lancet Haematol 3:e58–e59. https://doi.org/10.1016/j.
lancethaematol.2014.04.019.

25. Cui X, Lee R, Adler SP, McCoy MA. 2013. Antibody inhibition of human cytomegalovirus spread in epithelial cell cultures. J Virol Methods 192:
44–50. https://doi.org/10.1016/j.jviromet.2013.04.015.

26. Cui X, Meza BP, Adler SP, McCoy MA. 2008. Cytomegalovirus vaccines fail

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to induce epithelial entry neutralizing antibodies comparable to natural infection. *Vaccine* 26:5760–5766. https://doi.org/10.1016/j.vaccine.2008.07.092.

40. Saccoccio FM, Gallagher MK, Adler SP, McVoy MA. 2011. Neutralizing activity of saliva against cytomegalovirus. *Clin Vaccin Immunol* 18:1536–1542. https://doi.org/10.1128/CVI.05128-11.

41. Saccoccio FM, Sauer AL, Cui X, Armstrong AE, Habib el-SE, Johnson DC, Ryckman BJ, Klingelhutz AJ, Adler SP, McVoy MA. 2011. Peptides from cytomegalovirus UL130 and UL131 proteins induce high titer antibodies that block viral entry into mucosal epithelial cells. *Vaccine* 29:2705–2711. https://doi.org/10.1016/j.vaccine.2011.01.079.

42. Shedlock DJ, Talbott KT, Wu SJ, Wilson CM, Muthumani K, Boyer JD, Sardesai NY, Awasthi S, Weiner DB. 2012. Vaccination with synthetic constructs expressing cytomegalovirus immunogens is highly T cell immunogenic in mice. *Hum Vaccin Immunother* 8:1668–1681. https://doi.org/10.4161/hv.22447.

43. Schleiss MR, Bierle CJ, Swanson EC, McVoy MA, Wang JB, Al-Mahdi Z, Geballe AP. 2015. Vaccination with a live attenuated cytomegalovirus devoid of a protein kinase R inhibitory gene results in reduced maternal viremia and improved pregnancy outcome in a guinea pig congenital infection model. *J Virol* 89:9727–9738. https://doi.org/10.1128/JVI.01419-15.

44. Schäfer H, Kliem G, Kropp B, Burger R. 2007. Monoclonal antibodies to guinea pig interferon-gamma: tools for cytokine detection and neutralization. *J Immunol Methods* 328:106–117. https://doi.org/10.1016/j.jim.2007.08.012.