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Comparison of vaccination with rhesus CMV (RhCMV) soluble gB with a RhCMV replication-defective virus deleted for MHC class I immune evasion genes in a RhCMV challenge model

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ABSTRACT

A human cytomegalovirus (HCMV) vaccine to prevent infection and/or reduce disease associated with congenital infection or visceral disease in transplant recipients is a high priority, but has remained elusive. We created a disabled infectious single cycle rhesus CMV (RhCMV) deleted for glycoprotein L (gL) and the MHC class I immune evasion genes Rh178 and Rh182-189, and restored its epithelial cell tropism by inserting the Rh128-131A genes. The resulting virus, RhCMVRΔgL/178/182–189, was used to vaccinate rhesus monkeys intramuscularly and was compared with vaccination of animals with soluble RhCMV glycoprotein B (gB) in alum/monophosphoryl lipid A or with PBS as a control. At 4 weeks after the second vaccination, an increased frequency of RhCMV-specific CD8 T cells was detected in animals vaccinated with the RhCMVRΔgL/178/182–189 vaccine compared to animals vaccinated with soluble gB. In contrast, monkeys vaccinated with soluble gB had 20-fold higher gB antibody titers than animals vaccinated with RhCMVRΔgL/178/182–189. Titers of neutralizing antibody to RhCMV infection of fibroblasts were higher in animals vaccinated with gB compared with RhCMVRΔgL/178/182–189. Following vaccination, monkeys were challenged subcutaneously with RhCMV UCD59, a low passage virus propagated in monkey kidney epithelial cells. All animals became infected after challenge; however, the frequency of RhCMV detection in the blood was reduced in monkeys vaccinated with soluble gB compared with those vaccinated with RhCMVRΔgL/178/182–189. The frequency of challenge virus shedding in the urine and saliva and the RhCMV copy number shed at these sites was not different in animals vaccinated with RhCMVRΔgL/178/182–189 or soluble gB compared with those that received PBS before challenge. Although the RhCMVRΔgL/178/182–189 vaccine was superior in inducing cellular immunity to RhCMV, it induced lower titers of neutralizing antibody and antibody to gB than the soluble gB vaccine; after challenge, animals vaccinated with soluble gB had a lower frequency of virus detection in the blood than those vaccinated with RhCMVRΔgL/178/182–189.

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1. Introduction

Human cytomegalovirus (HCMV) infection of young children and adults is common and often asymptomatic. Occasionally a mononucleosis-like syndrome can accompany primary infection in adolescents and adults. HCMV infection of pregnant women is the most prevalent infectious cause of congenital disease worldwide [1]. Complications associated with congenital HCMV range from sensorineural hearing loss, optic atrophy, and intrauterine encephalitis to death. A recent report suggests that in utero infections with CMV is linked to development of childhood acute lymphoblastic leukemia [2]. The risk of congenital HCMV is generally thought to be lower in women with prior exposure to HCMV and preexisting immunity to the virus than in those with primary infection during pregnancy, although there is controversy about this in the field [3–5]. HCMV also causes severe disease in solid organ and hematopoietic stem cell transplant recipients. These patients can develop severe pulmonary, gastrointestinal, and hepatic disease, while AIDS patients can develop retinitis, esophagitis, colitis, and central nervous system disease. Patients with congenital immunodeficiencies can also develop life-threatening HCMV disease.

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HCMV is shed in the saliva and primary infection with the virus usually occurs through the oral mucosa. Epithelial cells are the most common site of initial virus infection. In patients infected during blood transfusion, HCMV infects monocytes and/or endothelial cells. HCMV contains multiple glycoproteins in its envelope and different combinations of glycoproteins are important for entry into different cells types. Epithelial cell infection requires glycoprotein B (gB) and the pentameric glycoprotein complex of gH/gL, UL128, UL130, and UL131A, while infection of fibroblasts requires gB and gH/gL or gH/gL/gO [6]. Recently gH/gL/gO was shown to be required for fusion of virus to both epithelial cells and fibroblasts [7]. The difference in glycoprotein complexes required for entry of HCMV into specific cell types is also reflected in the targets of neutralizing antibodies that inhibit infection for each specific cell type. HCMV gB is the primary target for neutralizing antibodies that prevent fibroblast infection, while the pentameric complex is the major target for neutralizing antibodies that prevent epithelial cell infection [8].

HCMV also contains a large number of proteins that it uses to modulate the host immune response. These include viral proteins that inhibit presentation of MHC class I and class II on the surface of virus-infected cells [9], inhibit activity of NK cells [10], block apoptosis [11], or mimic chemokines and chemokine receptors [12].

An effective vaccine for HCMV has been elusive to date. The vaccine furthest along in clinical trials is soluble HCMV gB adjuvanted with MF59. This vaccine reduced HCMV infection by 50% in women who were vaccinated within one year after giving birth [13] and reduced virus infection in adolescent females [14]. The gB vaccine also reduced the duration of viremia and the number of days of antiviral therapy required in HCMV seronegative solid organ transplant recipients that received transplants from HCMV positive donors [15]. Other HCMV vaccines under development include DNA vaccines that express HCMV gB and pp65 [16], a conditional replication-defective vaccine [17], chimeric live attenuated vaccines [18], and a vaccine that contain a pentameric glycoprotein complex [19].

Infection of rhesus macaques with rhesus CMV (RhCMV) is a good animal model for HCMV. Like HCMV, RhCMV is ubiquitous in its host population, most infected animals are asymptomatic and have lifelong shedding of virus in saliva, immunosuppressed animals can develop disseminated disease, and vertical transmission can result in congenital infection and birth defects [20]. Furthermore, RhCMV contains orthologs for most of the genes in HCMV including each of the glycoproteins that are targets of neutralizing antibodies. Infection of RhCMV seronegative rhesus macaques with a low passage epithelial cell tropic RhCMV strain results in lifelong infection and shedding similar to that observed with natural infection [21].

We developed a RhCMV vaccine that is a disabled infectious single cycle virus. We repaired the pentameric complex so that the virus could infect multiple cell types including fibroblasts and epithelial cells, but not spread from cell to cell. We also deleted the RhCMV genes that inhibit MHC class I presentation to try to increase presentation of viral proteins through MHC on infected cells. We vaccinated rhesus macaques with this vaccine or a soluble RhCMV gB vaccine and subsequently challenged the animals with wild-type virus.

2. Results

2.1. Construction of a defective infectious single cycle virus

Previously we constructed a RhCMV bacterial artificial chromosome (BAC) DNA deleted for RhCMV glycoprotein L (gL) termed RhCMVΔgL BAC [22]. We mutated RhCMVΔgL BAC so that the MHC class I immune evasion genes were deleted and the epithelial cell tropism genes were restored. HCMV encodes four genes, US2, US3, US6, and US11, which inhibit MHC class I presentation. RhCMV encodes orthologs, Rh182, Rh184, Rh185, and Rh189, of each of the HCMV proteins as well as a unique protein Rh178 that prevents translation of new MHC class I heavy chains [23,24]. Collectively, these RhCMV genes allow infected cells to evade recognition by cytotoxic T-cells [25], but leaves the infected cells susceptible to killing by NK cells, which lyse cells that lack class I molecules.

The Rh182-189 gene complex was deleted from the RhCMVΔgL BAC using the galactose kinase K recombineering protocol [26]. Rh182-189 was replaced with a galK cassette, and the galK cassette was then deleted (Fig. 1A). Rh178 was replaced with a galK cassette that was left in the BAC. The subsequent BAC was transfected into telomerase-transformed rhesus fibroblasts expressing RhCMVgL, Telo-RF:RhgL cells, to produce RhCMVΔgL/178/182–189 and virus was propagated in Telo-RF:RhgL cells. Cells infected with RhCMV deleted for Rh182-189 and Rh178 showed expression of MHC Class 1 that was similar to uninfected cells and increased compared to cells infected with virus without deletion of these genes (Fig. 1B).

The UL/b' region of RhCMV encodes Rh128, 130, 131A that are critical for epithelial cell tropism [27]. RhCMVΔgL/178/182–189 was derived from cell culture passaged RhCMV 68.1 which lacks Rh128 and a portion of Rh130, and the remaining portion of UL130 and UL131A are rearranged in the cell culture passaged strain relative to the unpassaged virus [28]. Therefore, we inserted a cassette containing Rh128, Rh130 and Rh131A into RhCMVΔgL/178/182–189 so that the resulting virus could infect epithelial cells. RhCMV UL128-131A, flanked by 500 bp of homologous 68.1 DNA was amplified by PCR. Telo-RF:RhgL cells were cotransfected with the RhCMV ΔgL/178/182–189 BAC and the purified RhCMV UL128-131A PCR product and two weeks later CPE was observed and the supernatant transferred to ARPE-19:RhgL cells. The resulting virus in which epithelial cell tropism has been restored was termed RhCMVRΔgL/178/182–189. PCR of DNA with primers corresponding to the end of UL128 and the beginning of UL130 yielded a 200 bp band using DNA from cells infected with RhCMVRΔgL/178/182–189 or low passage epithelial cell tropic RhCMV UCD59, while no band was detected using DNA from cells infected with RhCMVΔgL, indicating that the UL128-131A cassette had been inserted into RhCMV ΔgL/178/182–189 (Fig. 2A). RhCMVRΔgL/178/182–189 formed plaques in ARPE-19 epithelial cells expressing gL (Fig. 2B), further confirming that RhUL128, RhUL130, and RhUL131A were functional in the repaired virus. Unrepaired virus did not infect epithelial cells. Plaques were visible in Telo-RF:RhgL cells infected with RhCMVRΔgL/178/182–189, but not Telo-RF cells that do not express Rh gL, which only showed single cells rounded up, indicating that the virus was unable to spread and form plaques.

2.2. Vaccination of rhesus macaques with soluble gB elicits higher gB antibody titers and higher neutralizing antibody titers than vaccination with RhCMVRΔgL/178/182–189

Groups of four RhCMV seronegative rhesus macaques were vaccinated intramuscularly with (a) 50 ug of soluble RhCMVgB (soluble gB) formulated in 800 ug of alum and 50 ug of monophosphoryl lipid A (MPL) adjuvant, (b) 10^5 PFU of RhCMVRΔgL/178/182–189, or (c) PBS as a control. Soluble RhCMV gB was used as a positive control since HCMV gB is the vaccine that is furthest along in clinical trials [11,12]. The soluble RhCMV gB contained the extracellular domain of gB (amino acids 1–680) with a mutation in the furin cleavage site so that only the full length

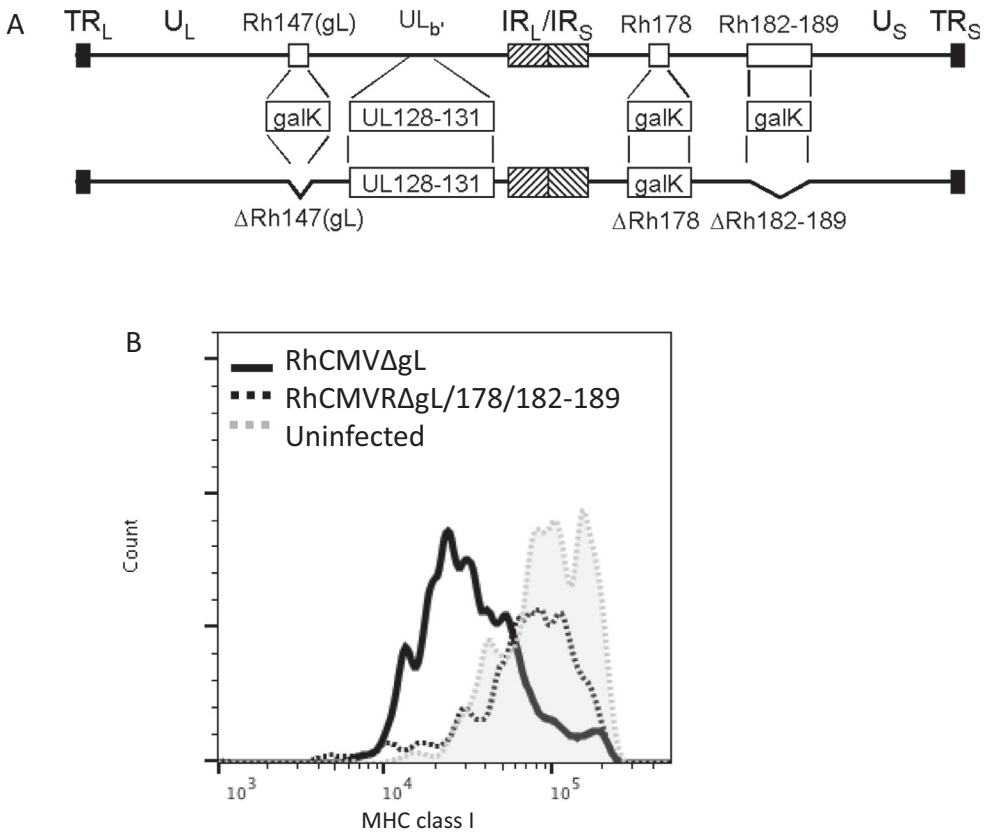

Fig. 1. Construction of vaccine virus and reduction in down-regulation of MHC class I in cells infected with RhCMVRΔgL/178/182–189. (A) Diagram of the RhCMV genome showing the sites of the deletions (gL, Rh178, Rh182-189) and insertion (UL128-131A) engineered into RhCMV and the PCR primers used to verify the mutations. (B) Rhesus fibroblasts expressing gL were infected with either RhCMVΔgL or RhCMVRΔgL/178/182–189 and stained with antibody to HLA MHC class I and RhCMV IE1 and analyzed by flow cytometry. RhCMVRΔgL/178/182–189 infected (RhCMV IE1-positive) cells had a 4.5-fold increase in MHC class I expression compared to RhCMVΔgL (which contains Rh182-189 and Rh178).

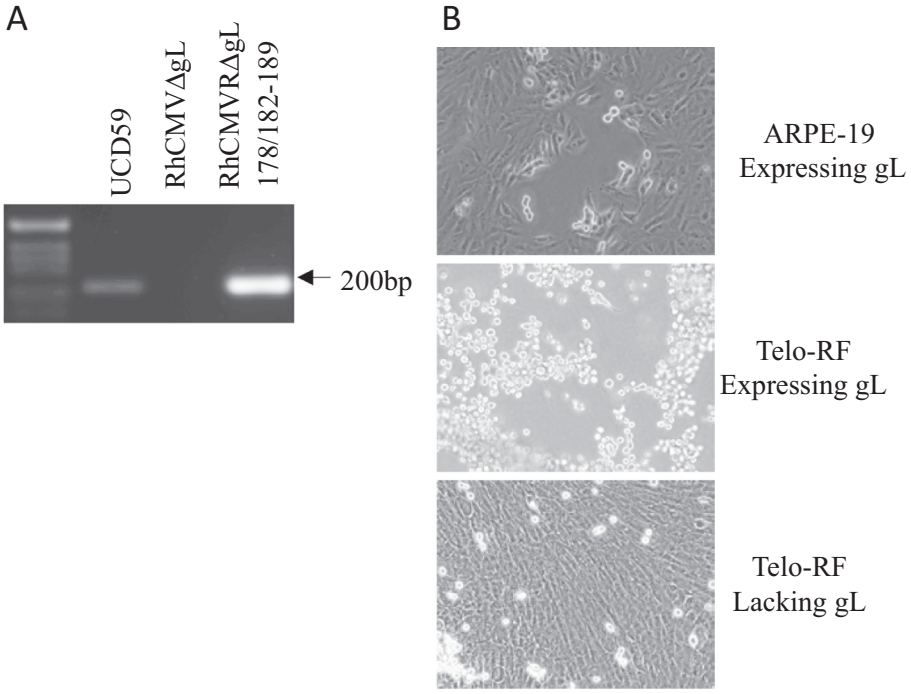

Fig. 2. Insertion of Rh128-131A into RhCMVΔgL restores epithelial cell tropism. (A) DNA from cells infected with UCD59 (low passage wild-type RhCMV), RhCMVΔgL, and RhCMVRΔgL/178/182–189 was amplified using primers in a region spanning UL128-UL130. A 200 bp band is detected in cells infected with UCD59 and RhCMVRΔgL/178/182–189, but not in cells infected with RhCMVΔgL, due to the deletion in the UL128-UL131A region in RhCMVΔgL. (B) ARPE-19 (epithelial) cells expressing gL, Telo-RF (fibroblast) cells expressing gL, or Telo-RF (fibroblast) cells not expressing gL were infected with RhCMVRΔgL/178/182–189 and cells were photographed.

(~135 kDa) extracellular domain was expressed (Fig. 3A and B). Alum/monophosphoryl lipid A (MPL) was used as an adjuvant with gB, since it produced nearly similar gB antibody titers in mice as gB adjuvanted with EM081, a squalene oil-in-water emulsion similar to MF59 (Fig. 3C); MF59 was the adjuvant used in phase 2 clinical trials with soluble gB [14,29]. Monkeys were vaccinated 3 times at weeks 0, 4 and 12 (Fig. 4A). Four weeks following the third vaccination, serum was collected and antibody responses were determined

All monkeys vaccinated with soluble gB or with RhCMVRΔgL/178/182–189 developed antibodies that immunoprecipitated gB. At 4 weeks after the third vaccination, monkeys that received soluble gB had significantly (20-fold) higher gB antibody titers than animals vaccinated with RhCMVRΔgL/178/182–189 ($p < 0.0001$, Fig. 4B). Neutralizing antibody titers, measured on rhesus fibroblasts, indicated that most vaccinated animals developed neutralizing antibodies 4 weeks after the third vaccination. Monkeys vaccinated with soluble gB had higher neutralization titers compared to animals vaccinated with RhCMVRΔgL/178/182–189 although the difference was not significant (Fig. 4C). Monkeys vaccinated with RhCMVRΔgL/178/182–189 did not develop epithelial cell neutralizing antibodies as determined by epithelial cell neutralization assay on monkey kidney epithelial (MKE) cells (data not shown).

2.3. Immunization of rhesus macaques with RhCMVRΔgL/178/182–189 induces RhCMV-specific CD8 T cell responses

Cellular immunity was measured in monkeys by analyzing the CD8 and CD4 T cell responses to RhCMV IE1 or pp65 four weeks after the second vaccination. Monkeys vaccinated with RhCMVRΔgL/178/182–189 had significantly higher RhCMV-specific CD8 T cells in the blood compared with animals that received soluble gB or PBS (Fig. 5A). The level of RhCMV-specific CD8 T cells varied widely in naturally infected animals. Vaccination with RhCMVRΔgL/178/182–189 did not elicit a RhCMV-specific CD4 T cell response in most of the animals at the time point that we tested (Fig. 5B).

2.4. Vaccination of rhesus macaques with soluble gB or RhCMVRΔgL/178/182–189 does not prevent infection or shedding

All animals were challenged subcutaneously with 10^6 PFU of RhCMV UCD59, a low passage epithelial cell tropic virus from naturally infected rhesus monkeys, at 8 weeks after the last vaccination. All of the animals became infected, as evidenced by shedding. Vaccination with soluble gB reduced the frequency of detectable viremia by 50% compared to PBS vaccinated monkeys ($p = .057$); vaccination with RhCMVRΔgL/178/182–189 had less effect on the frequency of viremia (Fig. 6). Vaccination with RhCMVRΔgL/178/182–189 or soluble gB did not reduce the percentage of virus shedding in the urine (Fig. 7A) or the number of virus DNA copies in the urine (Fig. 7B) compared with the PBS control group. At 12 weeks after challenge, 100% of animals vaccinated with RhCMVRΔgL/178/182–189, 75% given PBS, and 50% vaccinated with soluble gB shed virus in the urine. The percent of virus shedding in the saliva (Fig. 7C) and the number of virus DNA copies in the saliva (Fig. 7D) was not reduced after vaccination with RhCMVRΔgL/178/182–189 or soluble gB compared with the PBS control group.

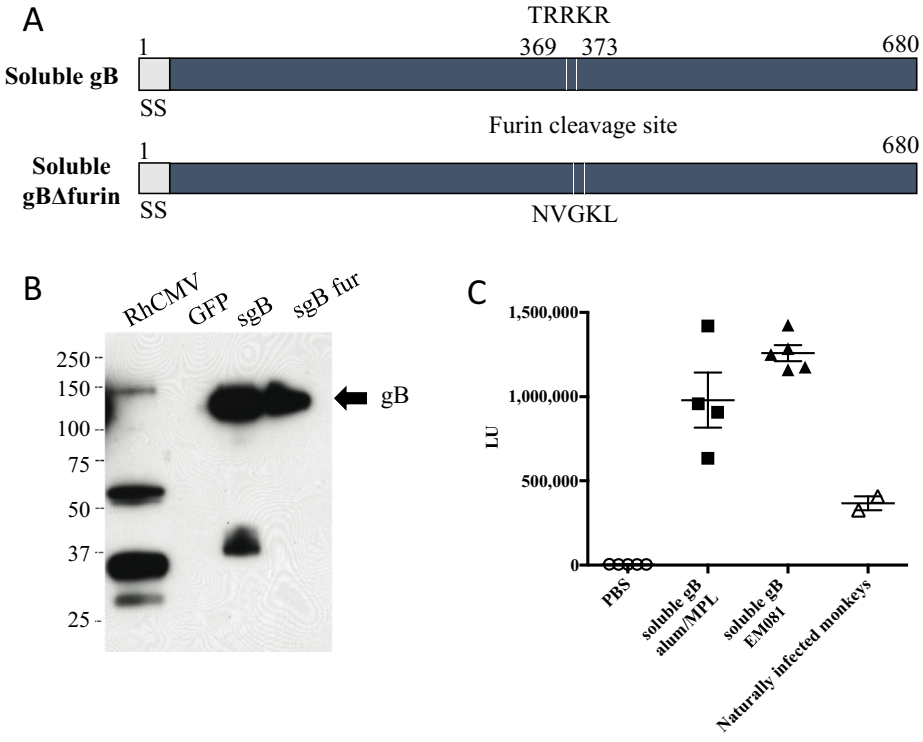

Fig. 3. Mutation of the furin cleavage site in soluble gB and purification of soluble gB. (A) Map of the extracellular portion of RhCMV gB with furin cleavage site (TRRKR, top line) and site of furin cleavage site mutations (NVGKL, lower line). (B) Western blot showing size of gB in Telo-RF cells infected with RhCMV, 293 T cells transfected with GFP, 293 T cells transfected with a plasmid expressing soluble gB (pND/gBΔ), and 293 T cells transfected with plasmid expressing soluble B with the furin cleavage site deleted (pND/gBΔfur). Lysates were run on an SDS-PAGE gel and immunoblotted with anti-HCMV gB AD-1 monoclonal antibody. Bands between 25 and 75 kDa are gB cleavage products. (C) Three groups of 4–5 mice were vaccinated with PBS, soluble gB adjuvanted with alum/MPL, or soluble gB adjuvanted with EM081, a squalene oil-in-water emulsion similar to MF59, 3 times 4 weeks apart. Two weeks following the last vaccination, sera were collected from the mice and gB antibody levels were detected using a gB luciferase immunoprecipitation system assay. LU indicates light units. Serum from monkeys naturally infected with RhCMV was used as a control.

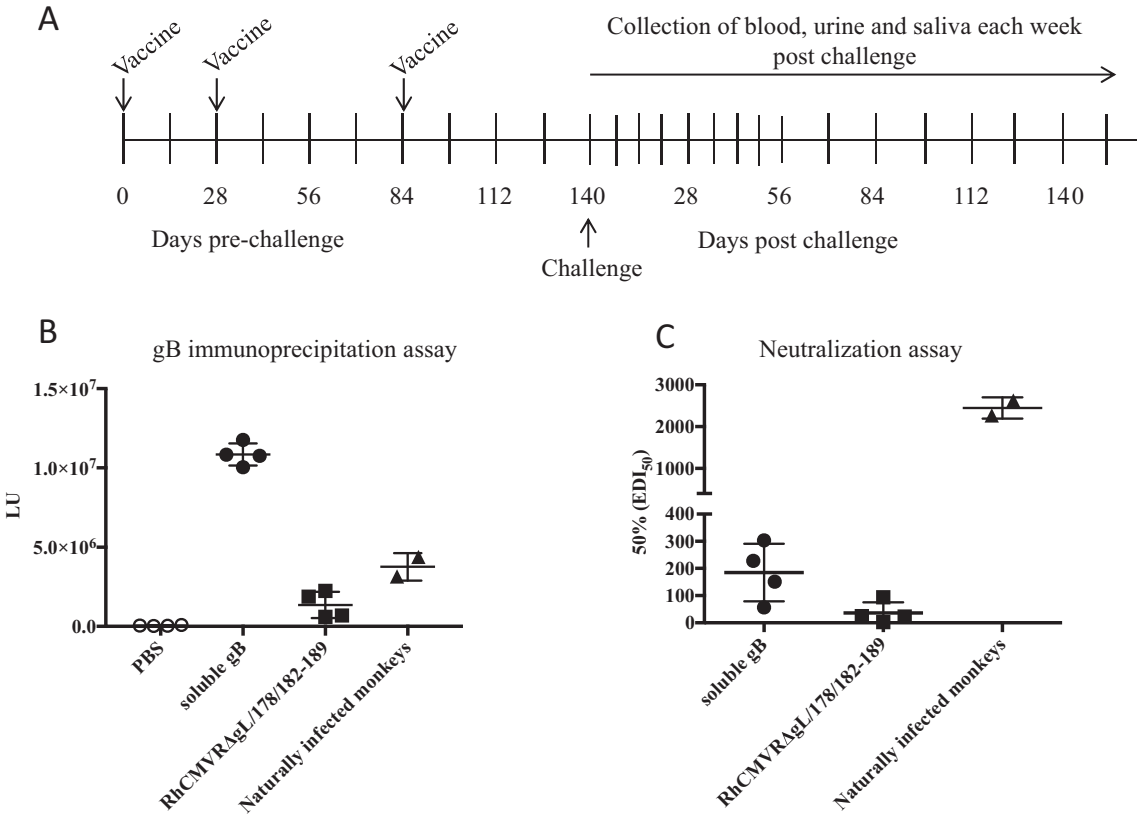

Fig. 4. Rhesus monkeys immunized with soluble gB have high titers of antibody to gB, but relatively low titers of antibody that neutralize fibroblast infection. (A) 12 rhesus monkeys were placed into 3 groups of 4 animals each. Group 1 was vaccinated with PBS, group 2 with soluble gB in alum/MPL adjuvant, and group 3 with RhCMVRΔgL/178/182–189. Vaccinations were given intramuscularly 3 times followed by subcutaneous challenge with 10^6 PFU of UCD59. (B) gB antibody levels were measured in sera from animals vaccinated with soluble gB, RhCMVRΔgL/178/182–189, or PBS, or from monkeys naturally infected with RhCMV by luciferase immunoprecipitation system assay and the number of light units (LU) corresponding to the titer of gB antibody at 4 weeks after the third vaccination is shown. (C) Neutralizing antibody titers from animals vaccinated with soluble gB, RhCMVRΔgL/178/182–189, or PBS, or from monkeys naturally infected with RhCMV were measured in fibroblasts 4 weeks after the third vaccination and the effective dilution of antibody that inhibits infectivity by 50% (EDI_{50}) is shown.

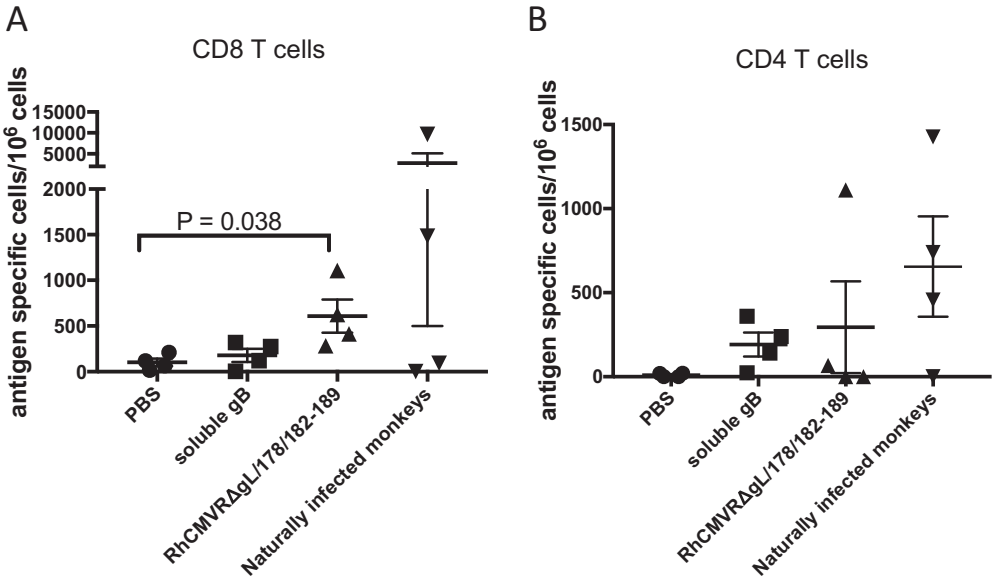

Fig. 5. Monkeys vaccinated with RhCMVRΔgL/178/182–189 have increased CD8 T cells that recognize RhCMV IE1 or pp65 compared with animals vaccinated with soluble gB. PBMCs from animals vaccinated with soluble gB, RhCMVRΔgL/178/182–189, or PBS or from monkeys naturally infected with RhCMV were stimulated with either IE1 or pp65 and CD8 (A) or CD4 (B) cells that produced both TNF-α and IFN-γ at 4 weeks after the second vaccination were quantified.

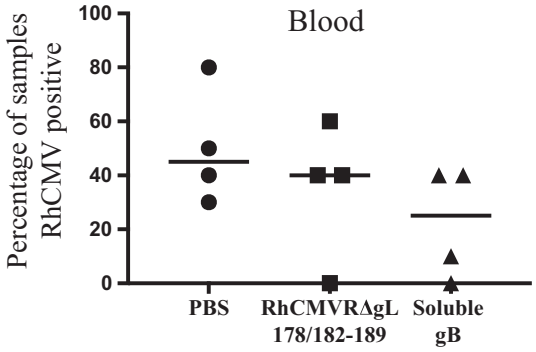

Fig. 6. Monkeys vaccinated with soluble gB have reduced frequency of RhCMV DNA detected in the plasma following challenge compared to PBS vaccinated animals. Monkeys vaccinated with soluble gB, RhCMVRΔgL/178/182–189, or PBS were challenged subcutaneously with 10^6 PFU of UCD59 8 weeks after the third vaccination. Following challenge, plasma was collected at time points shown in Fig. 4A and analyzed by real time PCR to determine the percentage of samples in each animal that were positive for RhCMV DNA. Each point represents a different animal. 10 plasma samples were collected from days 7 to 84 after challenge from each animal.

3. Discussion

We constructed a replication-defective virus, RhCMVRΔgL/178/182–189, deleted for gL and the viral genes that downregulate MHC class I expression (Rh178 and Rh182-189), and containing the viral genes (Rh128-131A) required for epithelial cell tropism. We vaccinated monkeys with the replication-defective RhCMV vaccine or with soluble RhCMV gB, the ortholog of the most effective CMV vaccine to date in clinical trials [14,29], and found that soluble gB, induced higher levels of gB antibody and neutralizing antibody in fibroblasts, while RhCMVRΔgL/178/182–189 induced higher RhCMV-specific CD8 T cell responses. When the monkeys were challenged, animals vaccinated with soluble gB had a lower frequency of viremia than those that received RhCMVRΔgL/178/182–189. Initial shedding in the urine, but not saliva, was slightly delayed in animals that received soluble gB compared with those receiving RhCMVRΔgL/178/182–189; however, the frequency of virus shedding in the saliva or urine and the RhCMV DNA copy number of virus shed at these sites was not reduced compared with animals that received PBS before challenge. Thus, while neither vaccine prevented infection or shedding, the soluble gB vaccine tended to reduce the frequency of virus detection in blood more effectively than the RhCMVRΔgL/178/182–189 vaccine.

RhCMVRΔgL/178/182–189 induced low titers of gB antibodies and fibroblast neutralizing antibodies and did not produce detectable epithelial cell neutralizing antibodies or RhCMV-specific CD4 T cell responses in most of the animals. In contrast, the soluble gB vaccine produced high levels of gB antibody and CD4 T cell responses, but low titers of fibroblast neutralizing antibodies and no epithelial neutralizing antibodies. The low antibody and CD4 T cell responses in animals vaccinated with the RhCMVRΔgL/178/182–189 vaccine could be due to the relatively low titer of nonreplicating virus (10^5 PFU) used for vaccination. In addition, while the RhCMVRΔgL/178/182–189 vaccine virus produced in complementing cells (expressing gL) contains the pentameric complex (gH, gL, UL128, UL130, and UL131A), when this virus subsequently infects non-complementing cells in animals, the lack of RhCMV gL will prevent formation of the pentameric complex in their cells. Thus, there would be limited opportunity to induce neutralizing antibody to the pentameric complex after

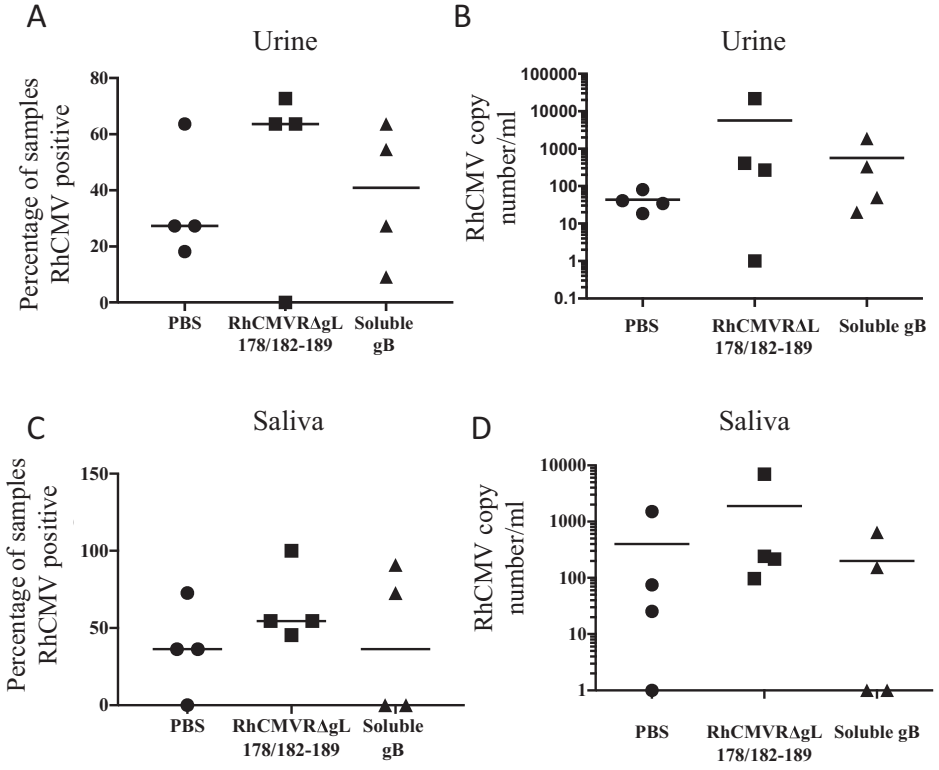

Fig. 7. Vaccination with soluble gB or RhCMVRΔgL/178/182–189 vaccine does not prevent shedding after challenge. RhCMV genome copy numbers were quantified by real time PCR in urine (A, B) and saliva (C, D) from 1 to 20 weeks after challenge of monkeys vaccinated with soluble gB, RhCMVRΔgL/178/182–189, or PBS. The percentage of shedding of virus in urine (A) or saliva (C) in animals after challenge is shown. 11 urine or saliva samples were collected from each animal; each point represents a different animal. The RhCMV copy number of virus per ml in the urine (B) or saliva (D) over time (area under the curve analysis) is shown. Each point represents a different animal.

viral replication. Antibodies to the pentameric complex have been shown to be the major target for neutralizing antibody in epithelial cells [19]. The low CD4 T cell response in our vaccine, compared with that observed in the human trials, may have been due the difference in adjuvant we used (alum/MPL) compared with that in the phase 2 trials (MF59).

The results described here parallel those of our prior experiments with a rhesus lymphocryptovirus vaccine to protect monkeys from the rhesus homolog of Epstein-Barr virus (EBV) [30]. Vaccination of animals with soluble rhesus lymphocryptovirus gp350 induced higher antibody titers, but lower virus-specific T cell responses to rhesus lymphocryptovirus than vaccination with vectors expressing EBV latency proteins and gp350. After challenge with wild-type virus, animals vaccinated with soluble rhesus lymphocryptovirus gp350 had a reduced frequency of viremia than those vaccinated with vectors expressing EBV latency proteins and gp350. Thus, both the rhesus cytomegalovirus and lymphocryptovirus experiments suggest that virus-induced antibodies that block the initial step of virus binding may have a critical role to reduce the frequency of viremia. In contrast, CD8 cellular immunity induced in animals vaccinated with RhCMVRΔgL/178/182–189 or vectors expressing EBV proteins had a much more limited effect on the frequency of viremia.

We found that vaccination with soluble gB or RhCMVRΔgL/178/182–189 did not prevent infection or significantly reduce shedding after monkeys were challenged with wild-type virus. There are several possibilities for the failure of the vaccines to prevent infection in the RhCMV challenge model. First, the monkeys were challenged subcutaneously which bypasses the normal route of infection, the mucosal epithelium, and it may be more difficult to protect animals with a non-natural site of challenge. In addition, most persons probably require multiple exposures with virus before infection is established [31], implying that the initial infection of mucosal epithelium is an inefficient event, while our animals all became infected after a single challenge dose. Modifying the route of challenge so that it represents natural infection through the mucosa may provide a more physiologic approach to study protection provided from vaccine. While a number of RhCMV vaccines have been tested in challenge models, none have been shown to prevent infection or shedding. All of these trials, like ours, used subcutaneous or in some cases intravenous challenge [32,33].

The correlate of protection needed for a prophylactic HCMV vaccine is unknown at present. While neutralizing antibody to HCMV has been assumed to be critical for protection from infection, two recent studies indicate that non-neutralizing antibody activities may be more important [34,35]. One of the complexities associated with development of a HCMV vaccine is that natural protection does not necessarily provide protection from reinfection of HCMV, however prior immunity to HCMV reduces the severity of disease associated with congenital HCMV infection and prevents a second case of HCMV infectious mononucleosis. It is possible that a vaccine that induces antibodies greater than the level obtained with natural infection would provide sterilizing immunity, based on the partial success of the gB vaccine in humans [14] or that it would reduce severity of disease after infection. The only licensed herpesvirus vaccine to prevent disease during primary infection is the varicella vaccine. While the correlate of protection for this vaccine is the level of antibody to varicella-zoster virus glycoproteins [36], the varicella vaccine is a live attenuated vaccine and it also induces strong T cell responses [37]. Both antibody and CD4 T cells have a role in protecting monkeys from congenital RhCMV [38,39]. Thus, either antibody or the combination of antibody and T cell responses may be required for an effective prophylactic herpesvirus vaccine.

4. Materials and methods

4.1. Cells and viruses

Telomerase transformed rhesus fibroblasts (Telo-RF cells, a gift from Peter Barry, University of California, Davis) and Telo-RF cells expressing RhCMV gL (Telo-RF:RhgL) [22] were maintained in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS). Human retinal pigmented epithelial cells (ARPE-19) were obtained from the American Type Culture Collection and maintained in DMEM/F12 (volume 1:1) supplemented with penicillin/streptomycin and 10% FBS. ARPE-19 cells expressing RhCMV gL were constructed by retroviral transduction as described for Telo-RF:RhgL [22]. MKE cells, a gift from Peter Barry, were maintained in DMEM/F12 supplemented with epithelial cell growth supplement (Sciencell), 1 mM sodium pyruvate, 25 mM HEPES, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2% FBS. MKE cells were grown on tissure culture dishes coated with collagen from Corning.

Challenge experiments were performed using RhCMV strain UCD59, a gift from Peter Barry [40,41]. RhCMV UCD59 was propagated in MKE cells to retain its epithelial cell tropism.

4.2. Animals

Balb/c mice (6–7 weeks old) were purchased from Charles River and rhesus macaques (*Macaca mulatta)* were purchased from the California National Primate Research Center. Monkeys were 1–2 years old and confirmed to be RhCMV seronegative using a RhCMV gB luciferase immunoprecipitation system (LIPS) assay [22] prior to vaccinations. The animal experiments were approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases and the studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

4.3. Plasmids

A plasmid expressing green fluorescent protein (GFP), pGL3:GFP, was obtained by excising the GFP gene from pEGFP:C2 [42] with XbaI and NheI and inserting the DNA into the corresponding sites of pgl3:control (Promega). pGL3:GFP contains the GFP gene under the control of the SV40 promoter and the bovine growth hormone polyadenylation sequence.

A plasmid expressing RhCMV gB with a deletion in the furin cleavage site, pND/gBΔFur, was constructed by mutating plasmid pND/gBΔ [43] (kindly provided by P. Barry) using a Quick Change Site-Directed Mutagenesis kit (Stratagene) and primers 5′-GGTTTTGTTAGAGTCCGATGCGGAATCAGTAGAAAGCTTTCCGACAGTACTACTGTTGGTATTGTTGTAAGC-3′ and 5′-GCTTACAACAATACCAACAGTAGTACTGTCGGAAAGCTTTCTACTGATTCCGCATCGGACTCTAACAAAACC-3′ (a novel HindIII site introduced by the mutation is underlined). The mutation was confirmed by digestion of the plasmid with HindIII and by sequencing of gB.

4.4. Construction of recombinant viruses

RhCMV deleted for gL and the MHC class I immune evasion genes Rh178 and Rh182-189, and containing the epithelial cell tropism genes UL128-131A, was constructed by mutating RhCMVΔgL BAC [22]. First, Rh182-189 was deleted using the galactokinase K (gal K) recombineering method [26]. A PCR product containing the *galK* gene with flanking regions corresponding to Rh182 and Rh189 was generated using plasmid pGalK (a gift from Neil

Copland) and primers 5′CACATGCCAGATA ATGGACAATGCGCAAAT CATTAAATGTAGTGTGCACACTGTCG**CCTGTTGACAATTAATCATCGG CA** and 5′GCCACC GCCTCACCCACCGATGTTTGAGATAGATCCTAA CAATATGAACACTG**TCAGCACTGTCCTGCTCCTT** (bold sequence corresponds to galK). *E. coli* strain SW102 containing RhCMVΔgL BAC was heat shocked at 42 °C fo 15 min and then electroporated with the PCR product containing *galK* with the Rh182 and Rh189 flanking sequences. After electroporation, the cells were plated onto M63 (minimal medium) plates with galactose as the sole carbon source and chloramphenicol. Bacterial colonies were screened for *galK* inserts by PCR and positive clones were plated onto MacConkey plates with galactose and chloramphenicol for selection of galK expression. Positive colonies were passaged on M63 plates with chloramphenicol and galactose to select clonal colonies.

To remove *galK* from the BAC, double stranded oligonucleotides 5′GCCAGATAATGGACAATGCGCAAATCATTAAATGTAGTGTGCACAC TGTCGCAGTGTTCATATTGTTAGGATCTATCTCAAACATCGGTGGGTG AGGCG 3′ and 5′CGCCTCACCCACCGATGTTTGAGATAG ATCCTAA CAATATGAACACTGCGACAGTGTGCACACTACATTTAATGATTTGCGC ATTGTCCATTATCTGGC-3′ containing RhCMV 182–189 sequences flanking the galK cassette were synthesized, annealed to each other, and electroporated into SW102 bacteria containing RhCMVΔgL/182–189/*galK* that had been heat shocked at 42 °C for 15 min. The bacteria were plated onto M63 minimal medium plates with 2-deoxy-galactose and glycerol as the carbon sources and chloramphenicol, colonies were selected and passaged on M63 plates with 2-deoxy-galactose, chloramphenicol, and glycerol to select clonal colonies.

Next Rh 178 was removed from the RhCMVΔgL/182–189 BAC. A PCR product containing the *galK* gene with flanking regions corresponding to Rh178 was generated using plasmid pGalK and the following primers: 5′ GGA CAC ACT TTC TGC TTC GCT CCC TCG GCC TGA CTG ATG ACT AGT CAT CGC ACG CCT CTT CCC GCC C**TC AGC ACT GTC CTG CTC CTT** and 5′ GTG TCC ATA CTG GCG AGT TTG TTC GTA TAA AAG TGT CGG ATG AAT GTG CGG CGC CAA CAC GCA GAC CC**C TGT TGA CAA TTA ATC ATC GGC A** (bold sequences correspond to galK). *E. coli* SW102 containing RhCMVΔgL/182–189 BAC was heat shocked and then electroporated with the PCR product containing *galK* with the Rh178 flanking sequences and bacterial colonies containg galK gene were selected as described above. GalK was not removed from the Rh178 mutation.

Next, the RhCMV UL128-131A genes were inserted into RhCMVΔgL/Δ178/Δ182-189 by homologous recombination. RhCMV UL128-131A, flanked by 500 bp of homologous 68.1 DNA were amplified by PCR using primers 5′-TAGCATCAACGTCACA GAAGT-3′ and 5′-TCTAGGGCGAGTAAGATGCG-3′. Telo-RF:RhgL cells were transfected with RhCMVΔgL/Δ178/Δ182-189 BAC DNA and gel purified PCR product encoding RhCMV UL128-131A, flanked by 500 bp of homologous 68.1 DNA using Lipofectamine 2000. Two weeks after transfection, CPE was observed and supernatant was transferred to fresh monolayers of ARPE-19:RhgL cells. A single plaque replicating on the ARPE-19:RhgL cells was observed and amplified to generate an epithelial cell tropic RhCMV lacking gL and Rh178, Rh182-189, termed RhCMVRΔgL/Δ178/Δ182-189.

GFP-expressing RhCMV was constructed for use in virus neutralization assays by replacing the Rh178 gene with a GFP expression cassette in the RhCMV 68.1.2 BAC. The Rh178 gene in 68.1.2 BAC [44] was replaced with galK as described above for RhCMVΔgL/Δ178/Δ182-189. To insert a GFP-expression cassette in place of Rh178, a SV40:GFP cassette was amplified from plasmid EGFP-puro [38] by PCR using primers 5′-GGACACACTTTCTGTTTC GCTCCCTCGGCCTGACTGATGACTAGTCATCGCACGCCTCTTCCCGCCC CGCTGTGGAATGTGTGTCA-3′ and 5′-GTGTCCATACTGGCGAGTTTG TTCGTATAAAAGTGTCGGATGAATGTGCGGCGCCAACACGCAGACCC TGCGATCTGCATCTCAAATAG-3′ (GFP-specific sequences are underlined) and introduced into the viral genome by recombineering. Replacement of Rh178 with the SV40:GFP cassette in RhCMV 68.1.2 Δ178:GFP BAC was confirmed by PCR and sequencing. To produce virus, RhCMV 68.1.2 Δ178:GFP BAC was transfected into Telo-RF cells using Lipofectamine 2000 (Invitrogen). Two weeks after transfection, supernatants were harvested, clarified by low speed centrifugation and transferred to ARPE-19 cells in order to maintain epithelial cell tropism. High titer stocks of RhCMV 68.1.2Δ178:GFP were grown by passing the virus once on Telo-RF cells.

4.5. MHC class I expression

Telo-RF cells expressing gL were infected with RhCMVΔgL or RhCMVRΔgL/Δ178/Δ182–189. 72 h after infection, cells were stained with MHC Class I antibody conjugated to APC (clone W6/32, Biolegend), fixed with 2% formaldehyde and permeabilized with 0.1% Trition X-100 for 4 min, followed by staining with polyclonal rabbit RhCMV IE1 antibody [45] (a gift from Peter Barry) and goat anti-rabbit IgG antibody conjugated to Alexa fluor 594 (Invitrogen). The level of MHC class I expression on Rh CMV IE1-positive cells was quantified by flow cytometry.

4.6. Protein expression and purification

To produce soluble RhCMV gB (soluble gB) 293 T cells plated in 10 cm dishes were transfected with 15 ug pND/gBΔFur using 100 ul of polyethylenimine (1.2 mg/ml; linear MW-25, Polysciences Inc.). Six days after transfection, supernatant was collected and filtered through 0.45 uM filters. RhCMV soluble gB was purified from 2 L of supernatant over an affinity column coated with mouse anti-HCMV gB AD-1 (kindly supplied by Bill Britt, University of Alabama) which cross reacts with RhCMV gB. RhCMV soluble gB was eluted with 0.1 M glycine pH 3, neutralized in 4 M Tris pH 8, concentrated, dialyzed with (citrate buffer), and stored at -80C.

4.7. Vaccination and challenge

Groups of 4–5 mice were vaccinated intramuscularly 3 times, 4 weeks apart, with soluble RhCMV gB adjuvanted either with alum (Alhydrogel, Brenntag) and MPL (Avanti Polar Lipids, Inc.) or with EM081- a squalene oil-in-water emulsion (similar to MF59) from the Infectious Diseases Research Institute, or with PBS. Blood was collected 2 weeks after the last vaccination.

RhCMV seronegative rhesus monkeys were immunized in one of four groups; 4 received RhCMVRΔgL/178/182–189, 4 received RhCMV soluble gB in alum/MPL, and 4 received PBS. Animals were vaccinated intramuscularly at week 0, 4, and 12 and were challenged subcutaneously at 4 sites in the back at week 20 with 10^6 PFU of RhCMV strain UCD59 (40, 41). Blood was collected on days –49, 0, 28, 56, 84, 112, and day 140 following vaccination, weekly following challenge until week 8 after challenge, and then biweekly until week 22. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradients and cryopreserved and plasma was frozen. Throat wash and urine samples were collected weekly following challenge until week 8 after challenge, and then biweekly until week 22. Urine was collected by cystocentesis and centrifuged at 2880 × *g* for 5 min at 4 °C. All but 2 ml of the supernatant was removed and the pellet was resuspended in the remaining supernatant and divided into 1 ml aliquots and frozen. Throat washes were collected by adding 2 ml of PBS to the throat and then the fluid was aspirated back with a syringe. The fluid was divided into 1 ml aliquots, centrifuged at 2880 × *g* for

5 min at 4 °C, all but 0.1 ml of the supernatant was removed, and the pellet was resuspended in the remaining supernatant and frozen.

4.8. Measurement of RhCMV gB antibody

The luciferase immunoprecipitation system (LIPS) assay was used to measure antibody levels to RhCMV proteins as described previously [22]. Briefly, a plasmid expressing RhCMV gB was transfected into Cos-1 cells, whole cell lysates were prepared, and a 1:10 dilution of plasma in buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X–100), 40 μl of buffer A, and 50 μl of 1×10^7 light units (LU) of Cos-1 cell extract containing RhCMV gB, were incubated at room temperature in each well of a 96-well plate. One hour later, 7 μl of a 30% suspension of protein A/G beads was added to each well of a 96 well filter HTS plate (Millipore) and 100 μl of the antibody-antigen complexes was added to the filter plate, incubated 1 hr, and washed 10 times in protein A and twice in PBS. LUs were measured after adding coelenterazine substrate (Promega) using a Berthold LB 960 Centro microplate luminometer (Berthold Technologies). LU data used were the mean of two independent experiments and corrected for background LU values of Cos-1 cell extract added to protein A/G beads not incubated with plasma.

4.9. Neutralization assays

Virus neutralizing antibody was quantified using a GFP-based assay that measures the ability of antibody to block infection of RhCMV 68.1.2Δ178:GFP on Telo-RF cells. Plasma from monkeys was serially diluted in 2-fold steps (from 1:5 to $1:2.6 \times 10^6$) and 25 ul of diluted antibody was added to each well of a 96 well plate. RhCMV 68.1.2Δ178:GFP (25 ul) was added to each well and incubated for 2 h at 37 °C. Telo-RF cells in suspension (4×10^4 cells in 50 ul) were added to each well. Cells, virus, and antibody were incubated 48 hr and cells were collected by removing media, adding 50 ul of TrypLE trypsin (Life Technologies) to the cells, and incubating for 20 min at 37 °C. 50 ul of 2.8% paraformaldehyde was added to fixed cells and GFP-positive cells (an indication of RhCMV infection) were measured using an Accuri C6 flow cytometer (BD Biosciences). Three wells of the 96 well plate were infected with virus alone as a positive control, and 3 wells were left uninfected as a negative control in each 96 well plate. The effective dilution of antibody that inhibited infectivity by 50% (EDI_{50}), based on the reduction of GFP-positive cells, was calculated by regression analysis.

Epithelial cell neutralization assay was done using MKE cells with epithelial cell tropic RhCMV UCD52. Two days after infection cells were stained with antibody to RhCMV IE1 (a gift from Peter Barry).

4.10. Cellular immunity

133 RhCMV pp65 overlapping peptides ([21], Mimotopes) were dissolved in DMSO at 53.2 mg/ml, pooled at 0.4 mg/ml, and frozen at −80 °C. 135 RhCMV IE1 overlapping peptides ([21], Mimotopes) were reconstituted in DMSO at 54 mg/ml, pooled at 0.4 mg/ml, and frozen. Cryopreserved rhesus PBMCs were thawed and rested overnight in RPMI medium with penicillin, streptomycin, 5% FBS, and IL-2 (10 U/mL, NCI repository). Cells (2×10^6/mL) were added to 96 well plates and stimulated with pooled RhCMV IE1 or pp65 peptides at 1 ug/ml for 1 hr at 37 °C in 5% CO_2, and 1 uM monensin was added for an additional 5 hr. Other aliquots of cells were left unstimulated or stimulated with PMA and ionomycin. Cells were washed and surface stained with CD4-PE (OKT4, Biolegend) and CD8-FITC (RPA-T8, BioLegend) for 20 min at room temperature. Cells were washed, incubated with cytofix/cytoperm (BD Pharmingen) for 30 min at 4 °C, washed, stained with anti-IFN-γ-APC (B27, BioLegend) and anti-TNF-α-PerCp (MaB11, BioLegend) for 1 hr at 4 °C. The cells were then washed, resuspended in FACS buffer, and analyzed on an Accuri C6 cytometer. Data were analyzed using Prism software.

4.11. Quantitative real-time PCR and primers

gB forward and reverse primers TGCGTACTATGGAAGAGACAATGC and ACATCTGGCCGTTCAAAAAAA, respectively, and a gB probe FAM/CCAGAAGTTGCGCATCCGCTTGTA/BLK_FQ were used for PCR and real-time PCR. Rh128-130 forward and reverse primers AGCAGGATTTGGCAAGAAGA and CGAAGCCAACTGTCACAAAA, respectively, were used to test that RhCMVRΔgL/Δ178/Δ182-189 was repaired for the Rh 128–130 region. GalK forward and reverse primers CCATTGTCGCACATGAAAAC and CTGCTTCCTGACCGTTAAGC, respectively, were used to detect insertion of GalK into RhCMV.

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