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Intranasal Immunization with Recombinant Vesicular Stomatitis Virus Expressing Murine Cytomegalovirus Glycoprotein B Induces Humoral and Cellular Immunity

Steven R Wilson,[1,*] Jean H Wilson,[1] Linda Buonocore,[2] Amy Palin,[2,†] John K Rose,[2] and Jon D Reuter[1,††]

Cytomegalovirus is a leading cause of morbidity and mortality among neonatal and immunocompromised patients. The use of vaccine prophylaxis continues to be an effective approach to reducing viral infections and their associated diseases. Murine cytomegalovirus (mCMV) has proven to be a valuable animal model in determining the efficacy of newly developed vaccine strategies in vivo. Live recombinant vesicular stomatitis viruses (rVSV) have successfully been used as vaccine vectors for several viruses to induce strong humoral and cellular immunity. We tested the ability of intranasal immunization with an rVSV expressing the major envelope protein of mCMV, glycoprotein B (gB), to protect against challenge with mCMV in a mouse model. rVSV–gB-infected cells showed strong cytoplasmic and cell surface expression of gB, and neutralizing antibodies to gB were present in mice after a single intranasal vaccination of VSV–gB. After challenge with mCMV, recovery of live virus and viral DNA was significantly reduced in immunized mice. In addition, primed splenocytes produced a $CD8^+$ IFNγ response to gB. The ability to induce an immune response to a gene product through mucosal vaccination with rVSV–gB represents a potentially effective approach to limiting CMV-induced disease.

Abbreviations: CMV, cytomegalovirus; gB, glycoprotein B; h, human; m, murine; rVSV, recombinant vesicular stomatitis virus

Cytomegaloviruses (CMVs) are double-stranded DNA viruses of the subfamily Betaherpesvirinae that can infect humans and animals and establish persistent and latent infections. Globally, human cytomegalovirus (hCMV) seropositivity is estimated to be 40% to 100%, and infection with hCMV is recognized as a leading cause of morbidity and mortality in susceptible populations.[28,35] Associated with clinical syndromes in humans for greater than a century,[68] CMV continues to be a leading cause of congenital viral infections in the United States, occurring in 0.4% to 2.3% of all live births with 10% of infected neonates exhibiting symptomatology in the form of mental retardation, visual impairment, or sensorineural hearing loss.[19,67,89] Acquired infections in children and adults with competent immune systems are typically asymptomatic, but with the advent of allograft organ transplantation, hCMV has become recognized as an important opportunistic pathogen.[27,70,75,85] In patients with impaired cellular immunity due to immunosuppressive drug therapy or AIDS, hCMV can lead to serious complications, including retinitis, fatal pneumonia, and encephalitis.[4,15,48,81,86,87,88]

The most effective means of providing protection against virus dissemination and disease after hCMV infection may depend on the patient population at risk. Vaccine prophylaxis is considered among the most economical and effective strategies for reducing transmission or lessening clinical disease associated with viral infections. CMV is a strict species-specific herpesvirus, and in vivo methods to uncover protective strategies against hCMV require the use of animal models incorporating mouse, rat, guinea pig, swine, and nonhuman primate CMVs.[77,78] For several decades, murine CMV (mCMV) has been used as an animal model to study the biology, pathogenesis, immunology, and immunoprophylaxis of CMVs.[3,14,37,38,44,61] This model has proven valuable in understanding the biology and immunology of hCMV because of its considerable gene sequence homology with hCMV and similarities in pathology.[29,58] As with other herpesviruses, mCMV infection results in the establishment of viral latency and reactivation after immunosuppression.[2,26] In addition, the immune responses to mCMV are similar to those seen in humans after hCMV infection.[84]

Because of their ability to induce strong intracellular synthesis and extracellular expression of viral antigens for extended periods of time, live-virus vaccines offer a means to produce robust immunity against these agents. Several vaccines against mCMV using live viral vectors have been developed, yet an ideal candidate capable of inducing persistent protective immunity has been elusive.[17,56,57,82,83] Recombinant vesicular stomatitis virus (rVSV) has been used successfully as an antigen-expression system and vaccine vector for both RNA and DNA viral proteins.[36,39,49,65,71,72,74] VSV shows little pathogenicity in infected humans, and recombinant forms are attenuated relative to wild-type VSV.[71] As a re-

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[1]Section of Comparative Medicine, [2]Department of Pathology, Yale University School of Medicine, New Haven, CT
[†]Present address: Program in Immunology, Stanford University, Stanford, CA
[††]Present address: The Salk Institute for Biologic Studies, La Jolla, CA
[*]Corresponding author. Email: steven.r.wilson@yale.edu

combinant vaccine vector, VSV can accommodate large inserts, induces strong humoral and cellular immunity, and can infect through mucosal surfaces, producing strong systemic and possibly local mucosal immunity. These attributes make rVSV an appealing candidate in developing a CMV vaccine, because the nasal, oral, and genital mucosae are natural routes for CMV infection.[65,72]

Mucosal immunization of mice with rVSVs expressing mCMV proteins may provide a model for altering CMV infection and preventing dissemination of virus. The CMV envelope glycoprotein B (gB) is the primary target for neutralizing antibodies produced in response to natural infection and therefore may serve as an effective antigen to alter host immunity.[11-13] However, vaccines successful in inducing gB-specific neutralizing antibodies have shown limited ability to induce a CTL response, limiting protection against disease.[5,50,57,83] Using a rVSV vector expressing mCMV glycoprotein B (VSV–gB), we developed and tested a vaccine candidate that not only produced significant levels of neutralizing antibodies in comparison to controls but also generated $CD8^+$-mediated cytokine production in vivo; crucial for the clearance of virus-infected cells. Lastly, we examined whether intranasal immunization with VSV–gB would provide a protective effect against mCMV challenge.

Materials and Methods

Mice. Six-week-old male Crl:CD1 (virus propagation) and 7- to 8-wk-old female BALB/cAnNCrl mice (immunization studies), specified to be free of murine viruses including mCMV, pathogenic bacteria, and parasites, were obtained from Charles River Laboratories (Wilmington, MA). All mice were acclimated for 3 to 5 d before experimental manipulation and housed at a temperature of 22 to 24 °C, humidity of 40% to 60%, and a 12:12-h light-dark cycle in a facility fully accredited by AAALAC International. Experimental and control mice were housed separately on corncob bedding (Harlan Teklad 7092, Indianapolis, IN) in static polycarbonate cages with filtered tops. Ante mortem manipulations were performed in a class IIA biological safety cabinet using standard microisolation techniques. Before experimental manipulation, random mice were verified by indirect immunofluorescent assay (described later) to be free of antibodies to mCMV. All procedures were performed after approval from the Yale University Institutional Animal Care and Use Committee.

Plasmid construction and recovery of recombinant virus. The gB gene was PCR-amplified from the pCMV-int-BL-gB clone (a gift from D Spector, University of California, San Diego) by using $Vent_R$ polymerase (New England Biolabs, Ipswich, MA), the upstream primer 5′GAT CGC CCT AGG AAA ATG TCA AGA AGA AAC GAA AGA GG3′, and the downstream primer 5′GCC TAG GTC AGT ACT CGA AAT CGG AGT C3′ to introduce *Avr*II endonuclease restriction sites (underlined) at both ends of the target fragment to allow cloning into the full-length VSV plasmid pVSVXN2.[80] The gB gene PCR product and full-length VSV plasmid were cleaved with *Avr*II and *Nhe*I (New England Biolabs, Ipswich, MA), respectively, before ligation of the gB gene into the *Nhe*I cloning site located between the genes encoding the G and L proteins of VSV. The inserted gene sequence for gB and its directionality were verified (WM Keck Facility, Yale University).

Live VSV–gB was recovered on BHK21 baby hamster kidney cells (American Type Culture Collection, Manassas, VA) as described previously.[39] Briefly, BHK21 cells were infected with recombinant vaccinia virus, vTF7-3 expressing T7 RNA polymerase, at a multiplicity of infection of 10 and incubated for 1 h in serum-free DMEM containing 100 U of penicillin–streptomycin. Full-length pVSV–gB (10 µg) and support plasmids (pBS-N [3 µg], pBS-P [5 µg], pBS-L [1 µg], and pBS-G [4 µg]), all under the control of T7 promoters, were transfected into vaccinia virus-infected cells in serum-free DMEM containing Transfectace.[73] After 3 h, the medium was replaced with DMEM containing 5% FBS (Gemini Bioscience, Woodland, CA), and the cultures were incubated for 48 h. The supernatant was collected, filtered through a sterile 0.2-µm filter to remove vaccinia virus, and passaged in fresh BHK21 cells. The supernatant was collected 24 h after cytopathic effect was observed. Stocks of virus were prepared from individual plaques grown on BHK21 cells and were stored at –80 °C.

Confirmation of protein expression after infection with VSV–gB. For metabolic labeling and endoglycosidase digestion of infected cells and virus, BHK21 cells in DMEM containing 5% FBS were infected with either wild-type rVSV or rVSV–gB at a multiplicity of infection of 20. After 4 to 6 h, medium was removed, and cells were washed with methionine-free DMEM. Labeled cell extracts were prepared by incubating cells at 37 °C for 1 h in 1 ml methionine-free DMEM containing 100 µCi [^{35}S]methionine. After removal of the medium, cells were washed in PBS and lysed with 500 µl detergent solution (1% Nonidet P40, 0.4% deoxycholate, 50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA) on ice for 5 min before transfer to a 1.5-ml microfuge tube. Protein extracts then were centrifuged for 2 min at 16,000 × *g* to remove nuclei and stored at –20 °C. For endoglycosidase digestion, protein extract was added to 1/10 volume of 10% NP-40 and 10X G7 buffer (0.5 M sodium phosphate [pH 7.5]) before incubating at 37 °C for 1 h with 1 µl of peptide *N*-glycosidase F. Digested and undigested protein extracts were fractionated by SDS-PAGE (10% acrylamide) and visualized by autoradiography.

Indirect immunofluorescent assay. To determine if cells infected with VSV–gB were capable of gB production and expression, 2×10^5 BHK21 cells in a total volume of 1.5 ml of DMEM containing 5% FBS were plated onto glass coverslips in a 35 mm dish. Cells were incubated overnight at 37 °C in 5% CO_2. Media was removed and cells were infected with 100 µl VSV–gB (5×10^8 pfu/ml) or 20 µl wild-type rVSV (6×10^8 pfu/ml) in a total volume of 500 µl serum-free DMEM. Infections were allowed to proceed for 5 h at 37 °C. Cells were washed twice with PBS and fixed at room temperature with 3% paraformaldehyde for 20 min. Fixed cells were washed twice with 10 mM glycine in PBS and stored at 4 °C. For staining, the cells were blocked and permeabilized for 5 min with 1% normal goat serum with 1% Triton. Cells were washed with PBS and incubated for 30 min at 37 °C with either a 1:250 dilution of a monoclonal mouse anti-gB antibody[83] or a 1:50 dilution of a polyclonal rabbit anti-VSV antibody.[65] Goat antimouse immunoglobulin-Cy3 (1:500; Chemicon, Temecula, CA) or goat antirabbit Alexa Fluor-350 conjugate (1:100; Molecular Probes, Eugene, OR) were used as secondary antibodies respectively. Coverslips were mounted by using Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence was recorded by using a fluorescence microscope with attached digital camera (Axioscope and AxioCam, Zeiss, Oberkochen, Germany).

Viruses. Murine CMV stock for challenge studies was generated by intraperitoneal inoculation of male Crl:CD1 mice with 5×10^4 pfu of the Smith strain of mCMV (originally from American Type

Culture Collection, Manassas, VA) in a volume of 100 µl. Thirteen days after inoculation, salivary glands were harvested, pooled, and homogenized in serum-free DMEM to produce a 10% w/v suspension. The homogenate was centrifuged for 5 min at 290 × *g* at 4 °C, and DMSO was added to the supernatant to provide a 10% v/v suspension for storage of aliquots at –70 °C.

A cell culture derived stock of the Smith strain of mCMV was propagated by inoculating a monolayer of 7×10^6 3T3 mouse fibroblast cells with 100 µl of the salivary gland homogenate. Cells were incubated in complete media—DMEM supplemented with 10% heat-inactivated FBS (Gibco certified, Gibco–BRL, Grand Island, NY), 100 µg/ml streptomycin, and 100 units/ml penicillin—at 37 °C in 5% CO_2 for 3 d until greater than 90% CPE was observed. Cells were manually dislodged from the flask surface, and the cell suspension was collected before undergoing 3 freeze–thaw cycles. The suspension was centrifuged for 5 min at 500 × *g* at 4 °C and the supernatant aliquoted for storage at –70 °C.

Titration of viral stocks and experimental samples. Titration of viral stocks and tissue samples was determined by standard viral plaque assays. Briefly, BHK21 (VSV–gB) or 3T3 (mCMV) were seeded onto 6-well (35 mm) plates in complete medium at a density to provide 80% confluent monolayers by the following morning. Logfold serial dilutions of viral stocks or samples were prepared in serum-free DMEM. Plated cells were washed in serum-free DMEM, inoculated in duplicate with 500 µl of each dilution, and incubated on a rocking platform at 37 °C for 60 min. After removal of viral dilutions, cells were overlaid with 2 ml of complete medium containing 1.2% SeaPlaque agarose (FMC BioProducts, Rockland, ME) and incubated at 37 °C in 5% CO_2 for 3 (VSV–gB) to 5 (mCMV) d. Plaques were fixed in neutral buffered formalin and stained for 30 min in modified Giemsa before being counted on an inverted microscope (CK2, Olympus, Tokyo, Japan) at 40× magnification.

Immunization/challenge studies. To verify that mice were mcMV-free as specified by the vendor, 100 µl of blood was collected from the retro-orbital sinus. Sera were separated and stored at –70 °C for IFA to confirm the absence of preexisting anti-gB antibodies. Cohorts of 5 mice were anesthetized with 20% isoflurane in propylene glycol (v/v) by using the open drop method.[30] One cohort was vaccinated intranasally with an inoculum of 5×10^6 pfu of VSV–gB in a volume of 25 µl of PBS. Control mice received either 4.75×10^6 pfu of wild-type VSV (VSV–egfp) in a volume of 25 µl of PBS or a similar volume of PBS intranasally. At 4 wk after immunization, prechallenge sera samples were collected and stored as described earlier for titration of anti-gB antibodies by immunofluorescent assay. Control and vaccinated mice were challenged with an intraperitoneal dose of 2.25×10^3 pfu of Smith strain mCMV in 100 µl PBS. Mice were observed daily for signs of clinical illness. At 10 d after viral challenge, mice were euthanized with CO_2 gas. Salivary glands, lungs and spleens were harvested, snap-frozen in liquid nitrogen, and stored at –70 °C. As described earlier, 10% tissue homogenates (w/v) were prepared in serum-free DMEM and stored as a suspension with 10% DMSO.

Tissue load of mCMV was determined by viral plaque assay and real-time PCR. Live mCMV in tissues was titered by using a standard plaque-forming assay on 3T3 cells as described earlier. For determination of viral DNA titers, DNA samples were prepared for PCR from 250 µl of the 10% tissue homogenates in 100 µl of extraction buffer by using a DNeasy tissue kit (Qiagen, Valencia, CA). The DNA concentration of each sample was determined by spectrophotometry at a wavelength of 260 nm. Sample DNA (500 ng) was added to a 25-µl reaction tube containing reagents from the QuantiTect SYBR green real-time PCR kit (Qiagen) and mCMV-gB primers, 5′CGA AAG AGG ATG TCG CTC C3′ (forward) and 5′CTC TTG TAC GGG TGT CTT CG3′ (reverse).[6] Real-time PCR and melting curve analysis were performed in triplicate using the DNA Engine Opticon 2 PCR system (MJ Research, Waltham, MA). Sample values are given relative to gB DNA standards obtained from serial dilutions of DNA extracted after amplification of the gB gene from pCMV-int-BL-gB.

Evaluation of humoral immunity. Indirect immunofluorescence and neutralization antibody assays were used for detection and titration of antibody to mCMV-gB. Sera samples were collected to evaluate antibody production 4 wk after vaccination and 10 d after viral challenge in the experimental and control groups. The A13 cell line (a gift from J Shanley, University of Connecticut), which constitutively expresses a truncated form of mCMV-gB, and 3T3 cells infected with the Smith strain of mCMV were used as cellular targets for immunofluorescence.[83] Cells were cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2 and harvested by trypsinization when the monolayers reached 80% confluency or exhibited signs of CPE. The cell suspensions were washed twice in serum-free DMEM, fixed in a freshly prepared 4% paraformaldehyde solution, and air-dried onto 12-well 6-mm microslides. Serial dilutions of sample sera from immunized and control mice were prepared in PBS containing 1% bovine serum albumin. Target cells were blocked and permeabilized for 15 min at room temperature in PBS containing 1% bovine serum albumin and 0.2% Triton and washed in PBS before addition of 25 µl of sample sera. After incubation at 37 °C for 1 h, cells were washed in PBS, and incubated with 25 µl of a goat antimouse immunoglobulin–Cy3 (1:500 in PBS containing 1% bovine serum albumin) secondary antibody for 30 to 45 min at 37 °C. Slides were washed in PBS and mounted in Vectashield (Vector Laboratories). Antibody titration was determined by rhodamine fluorescence using a Zeiss Axioscope with a Zeiss AxioCam digital camera.

The presence of virus neutralizing antibodies in prechallenge sera was determined by a modification of Gonczol's microneutralization plaque assay protocol.[20] Briefly, 2×10^4 3T3 cells in DMEM containing 10% fetal bovine serum were plated per well in a 96-well tissue culture plate and allowed to attach. Sample sera and pooled mCMV convalescent serum were diluted 1:10 in serum-free DMEM. Serum dilutions or medium alone were incubated with 2×10^3 pfu Smith strain mCMV for 1 h at 37 °C prior to the addition of 100 µl of the resultant mixture to 3T3 cells and incubation for 1 h at 37 °C. The serum–virus suspensions were aspirated from the cells and replaced with 200 µl of DMEM containing 10% fetal bovine serum. Cell cultures were maintained for 3 to 4 d at 37 °C in 5% CO_2. Cells then were fixed in 10% neutral buffered formalin and stained with Giemsa. Plaques were counted and results expressed as percentage plaque reduction compared with controls.

Evaluation of cellular immunity. Cytokine secretion and intracellular cytokine staining were used to determine the cellular immune response induced by vaccination with VSV–gB. Splenocytes were primed in vivo with a 25 µl volume of VSV–gB (5×10^6 pfu) or PBS administered intranasally. Because of the viscosity and volume of tissue homogenate necessary for infection, Smith strain mCMV (1×10^6 pfu) was inoculated intraperitoneally to ensure a uniform infective dose. After 8 d, mice were euthanized with

CO_2. Spleens were harvested, homogenized in ice cold Ca^{2+}-free, Mg^{2+}-free PBS and centrifuged for 10 min at 100 × *g*. Cells were resuspended in DMEM containing 10% fetal bovine serum at a final concentration of 2.5×10^6 cells/ml. In a 96-well tissue culture plate, 200 µl of cell suspension was plated per well with either 10 ng/ml phorbol myristate acetate and 40 ng/ml ionomycin (Sigma, St Louis, MO) or 50 µg/ml UV-inactivated recombinant mCMV–egfp.[64]

Intracellular cytokine staining was performed on in vivo stimulated splenocytes that had been cultured for 16 h at 37 °C in 5% CO_2. To prevent the secretion of cytokines, cells were incubated for an additional 8 h with the protein transport inhibitor brefeldin A (GolgiPlug BD Biosciences, Mississauga, ON Canada). Cells were washed and then resuspended in 50 µl of fluorochrome-conjugated rat antimouse monoclonal antibodies to cell surface molecules (1:50). Antibodies for detection of $CD4^+$ (Clone RM4-5) and $CD8^+$ (Clone 53- 6.7) cells were purchased from BD PharMingen (San Diego, CA). After a 30-min incubation period in dark on ice, cells were washed, fixed, and permeabilized by using Cytofix/Cytoperm kit reagents (BD Biosciences, San Jose, CA) according to manufacturer instructions prior to staining for IFNγ (Caltag Laboratories, Burlingame, CA). Labeled cells were analyzed by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA) capable of 4-color data collection. Data analysis was performed with FlowJo (Tree Star, Ashland, OR) cytometric analysis program.

Cytokine secretion was evaluated on cell culture supernatants collected after 72 h of incubation and assayed in duplicate for IFNγ by using an OptEIA enzyme-linked immunosorbent assay kit (BD Biosciences, San Diego, CA). Optical density was measured at 450 nm on a microplate absorbance reader (MRX Revelation, ThermoLabsystem, Franklin, MA).

Statistical analysis. Means, standard deviation, and standard error were determined for virus load in experimental and control groups. Differences between groups were evaluated by using analysis of variance. Paired Student *t* tests were performed on pre- and postchallenge antibody titers to evaluate humoral responses (NCSS, Kaysville, UT). *P* values less than 0.05 were considered statistically significant.

Results

Expression of mCMV-gB in rVSV. Recombinant viruses containing heterologous genes have shown the potential to act as vaccine and expression vectors. To explore the efficacy of such a vaccine in reducing infection and dissemination of mCMV to target tissues, we constructed and recovered rVSV–gB, which encodes the major envelope glycoprotein of mCMV. The gB gene was inserted between the genes encoding the L and G proteins of VSV (Figure 1 A). Before assessing the functionality of VSV–gB as a vaccine, we tested the ability of VSV–gB infected cells to express a specific protein product. Cell lysates were analyzed by SDS-PAGE for gB expression (Figure 1 B). Infection resulted in the production of the L, G, N, P, and M protein bands characteristic of wild-type rVSV infection as well as a gB band between the L and G proteins. To confirm that the sharp band representing expressed gB product was glycosolated, proteins were digested with endoglycosidase F prior to electrophoresis. The digested VSV G protein and gB migrated with electrophoretic patterns indicative of molecular weights lower than those of the glycosolated proteins. In vitro detection of surface and cytoplasmic expression of VSV–gB was confirmed by indirect immunofluorescence. After incubation with either wild-type rVSV or rVSV–gB, BHK21 cells expressed VSV proteins on their plasma membranes demonstrating successful infection with rVSV. In addition, rVSV–gB-infected cells expressed gB primarily on their cell surfaces, whereas binding of gB antibodies was not present on cells infected with wild-type rVSV (Figure 1 C).

Intranasal rVSV–gB immunization and response to mCMV challenge. To assess the efficacy of a mucosal rVSV–gB vaccine to protect against infection with mCMV, a single 25-µl intranasal dose of 5×10^6 pfu rVSV–gB was administered to BALB/c mice. Immunized mice exhibited minimal adverse effects, resulting in mildly ruffled fur without marked changes to behavior or appetite, as has been noted after infection with other VSV recombinants. None of the mice required veterinary intervention, and all continued to thrive throughout the study. Four weeks after immunization and 10 d after challenge, serum was collected from vaccinates and controls to determine anti-gB antibody development. Indirect immunofluorescent assays using a cell line expressing mCMV-gB demonstrated that 100% of vaccinated mice developed serum antibodies to gB in high titers, whereas sera from control mice vaccinated with either PBS or rVSV–egfp were antibody-negative (Table 1). Sera from vaccinates 10 d after challenge exhibited a marked rise in anti-gB titers. Sera from mice vaccinated with PBS or VSV vector alone continued to show no postchallenge affinity for gB, suggesting that the humoral responses to gB are specific to antigen expression and not due to nonspecific effects of the vector.

Serum samples collected before and 4 wk after immunization were examined for the presence of gB-binding and mCVM-neutralizing antibodies. Sera from unimmunized mice and polyclonal antibody derived from mCMV-infected mice were used as controls. Immunization with VSV–gB significantly ($P < 0.05$) induced neutralizing antibodies (Figure 2).

To determine protection provided by VSV-gB, a challenge dose of live mCMV was administered to mice 4 wk after immunization. The Smith strain of mCMV, heterologous from the K181 strain used to construct the vaccine, was injected intraperitoneally. Ten days later, mice were euthanized, and salivary gland, lung, and spleen were harvested to determine the level of live virus and viral DNA present in tissues. In the lungs, vaccinated mice showed significantly ($P < 0.05$) reduced viral titers compared with those of unvaccinated controls (Figure 3 A, B). PCR analysis of salivary gland and spleen homogenates and plaque assays of spleen did not show significant reduction of viral load in vaccinated mice. Control and vaccinated mice displayed some level of live virus recovery from each tested tissue indicating that protection from mCMV infection was incomplete after a single intranasal immunization with rVSV-gB.

Ability of VSV–gB to induce cellular immunity. In assessing the capacity for rVSV–gB to stimulate a cellular immune response, gB-primed splenocytes were assayed for intracellular staining and secretion of IFNγ. When splenocytes from rVSV–gB-immunized mice were cultured in the presence of UV-inactivated mCMV, IFNγ was secreted in elevated levels compared with those of controls (Figure 4). The subset of cells responsible for IFNγ production after rVSV–gB immunization was determined by culturing splenocytes for 24 h with UV-inactivated mCMV and staining with fluorochrome-tagged monoclonal antibodies for IFNγ, $CD4^+$, and $CD8^+$ before flow cytometric analysis (Figure 5). The presence

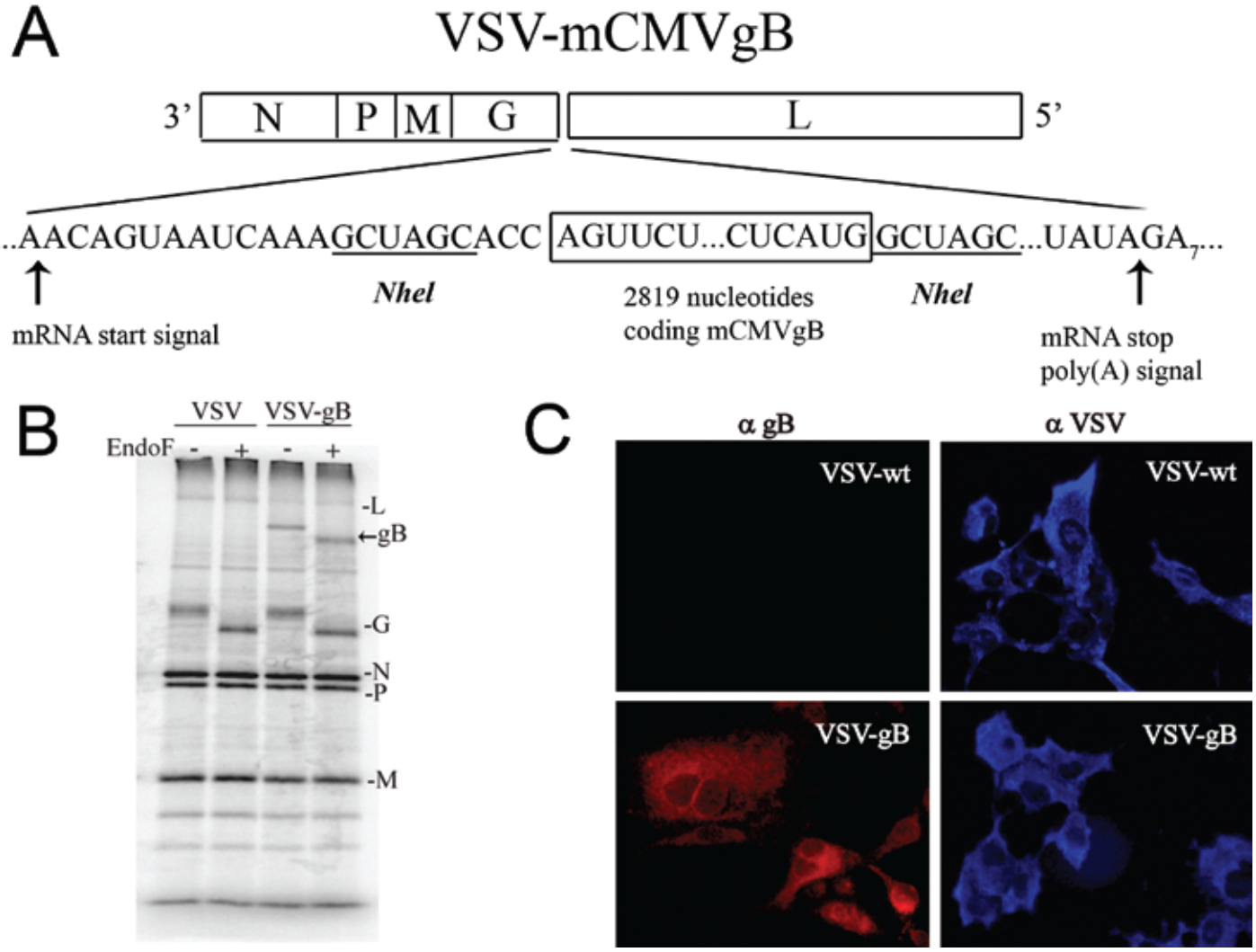

Figure 1. Recombinant VSV genome and protein expression. (A) Schematic representation of the rVSV–gB genome. The mCMV–gB gene insertion site and gene order is shown as the transcription of the negative-strand RNA in the 3′ to 5′ direction. *Nhe*1 labels indicate the restriction enzyme sites used for cloning the mCMV-gB gene at the DNA stage. (B) SDS-PAGE gel containing lysates of BHK cells infected with rVSV–gB or wild-type VSV and labeled with [^{35}S]methionine. Identified bands represent major VSV proteins and mCMV-gB. Lysates treated with *N*-glycosidase F (EndoF +) resulted in digestion of the glycosylation sites of the VSV G protein and mCMV glycoprotein B. (C) Immunofluorescence images of BHK cells infected with rVSV–gB or wild-type VSV. Upper and lower right panels illustrate infection and expression of VSV proteins in both recombinant virus cell cultures by using a rabbit polyclonal antibody directed against a recombinant VSV lacking the gB gene. Expression of gB is localized to the plasma membrane and cytoplasmic compartments and is seen only rVSV–gB-infected cells (lower left panel).

of mCMV antigen led to a marked increase in the total number of CD8$^+$ cells among splenocytes primed with rVSV–gB. The percentage of cells double-staining for IFNγ and CD8$^+$ was greater for cells primed with rVSV–gB than for PBS controls. No increase in IFNγ and CD4$^+$ positives was observed when spleen cells taken from VSV–gB immunized mice were cultured in the presence of mCMV antigen.

Discussion

The hallmark of a CMV infection in an immunocompetent host is an initial acute asymptomatic phase followed by the establishment of latency. Primary infection or reactivation of a latent infection in subjects with dysfunctional immune systems often leads to marked illness and mortality secondary to CMV pneumonia, encephalitis, and retinitis.[4,15,48,81,86,87,88] The complex processes of host immunity to CMV necessitate the activation of competent innate and adaptive arms of the immune system.[10]

The goals of immunization against CMV are to inhibit or reduce transmission and prevent disease during active infection through the induction of effective humoral and cellular responses. An ideal vaccine also would have the capacity to clear infection and prevent latency. The implementation of CMV vaccine strategies has included live attenuated viral vaccines, DNA vaccines, recombinant subunit vaccines, vectored subunit vaccines, and dense-body vaccines.[76] The use of antigenic targets of neutralizing antibodies and cytotoxic T-cell responses directed against hCMV have led to the development of vaccines providing varying degrees of protection against infection or disease. These methods have produced mixed results, with no superlative candidate.

Table 1. Antibody responses to gB

Intranasal immunization with	Mouse no.	Antibody titer to gB[a]		
		Before immunization	Before challenge[b]	After challenge[c]
VSV–gB	1	<1:10	1:7290	1:42,000
	2	<1:10	1:21870	1:84,000
	3	not done[d]	1:7290	1:42,000
	4	not done	1:7290	1:42,000
	5	not done	1:810	1:84,000
VSV–egfp	1	<1:10	<1:10	<1:10
	2	<1:10	<1:10	<1:10
	3	not done	<1:10	1:30
	4	not done	<1:10	<1:10
	5	not done	<1:10	<1:10
PBS (control)	1	<1:10	<1:10	<1:10
	2	not done	<1:10	<1:10
	3	not done	<1:10	1:30
	4	not done	<1:10	<1:10
	5	not done	<1:10	<1:10

[a]$P < 0.005$ (Student t test) between values for samples obtained before and after challenge.
[b]Immunofluorescent assay performed on sera taken 4 wk after immunization.
[c]Immunofluorescent assay performed on sera taken 10 d after challenge.
[d]Sera from representative mice of each cohort were tested to confirm the absence of preexisting antibodies to mCMV-gB

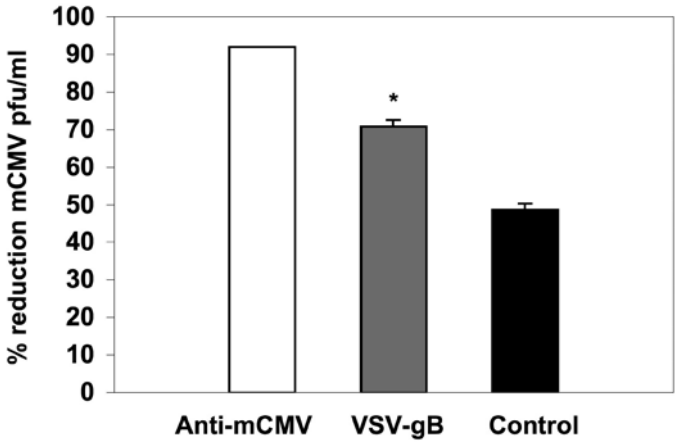

Figure 2. Immunization with VSV-gB produced a significant increase in mCMV neutralization antibodies. Serum was collected from BALB/c mice 4 wk after intranasal immunization with 5×10^6 pfu of VSV-gB or PBS. Pooled anti-mCMV serum from convalescent mice served as a positive control. Neutralizing activity was determined on 1:10 dilutions of sample sera and a polyclonal mCMV convalescent serum using a microneutralization assay. *, $P < 0.05$ compared with control value.

In this study, our aim was to develop a vaccine that resulted in robust stimulation of specific humoral and cellular responses against viral gB antigen. A live rVSV expressing murine CMV glycoprotein B was chosen based on biologic properties and targeted immunologic responses generated by rVSV. When administered across a mucosal surface, rVSV–gB induced robust humoral and cellular immune responses, as predicted from other animal models of human viral diseases and recombinant VSV expression vectors.[53,55,65,72,79]

Considered the major target of neutralizing antibodies acquired after natural infection, gB, the CMV envelope glycoprotein, was a logical choice for incorporation into our recombinant vaccine.[11,12,13] Attempts to use gB as an immunogen in the development of vaccines for hCMV have been successful in producing high antibody titers, but protection was incomplete.[1,5,9,50] We hypothesized that both humoral and cellular responses against gB would offer enhanced protection. A single dose of rVSV–gB, administered as a mucosal vaccine, induced a strong systemic antibody response against mCMV-gB within a period of 4 wk. When challenged with live mCMV, immunized mice exhibited a marked anamnestic humoral response with 3- to 4-fold increases in antibody titers. In neutralization assays, incubation of prechallenge sera from immunized mice with live mCMV led to reductions in the numbers of viral plaques, compared with those of control sera.

One of the earliest vaccines against mCMV, a vaccinia virus developed as an expression vector for mCMV-gB, resulted in production of complement-dependent neutralizing antibodies.[57] Others similarly demonstrated that immunization of mice with a replication-deficient adenovirus vector expressing mCMV-gB produced high levels of neutralizing antibodies but was unable to induce a detectable cellular immune response.[83] The induction of specific neutralizing antibodies in high titers alone is insufficient for effective and sustained protection against herpesviral infections.[16,82,83] In addition, recurrent mCMV infections in the presence of neutralizing antibodies indicate that the molecular interactions with the virus alone are unlikely to affect the control of viral reactivation. The importance of humoral immunity appears

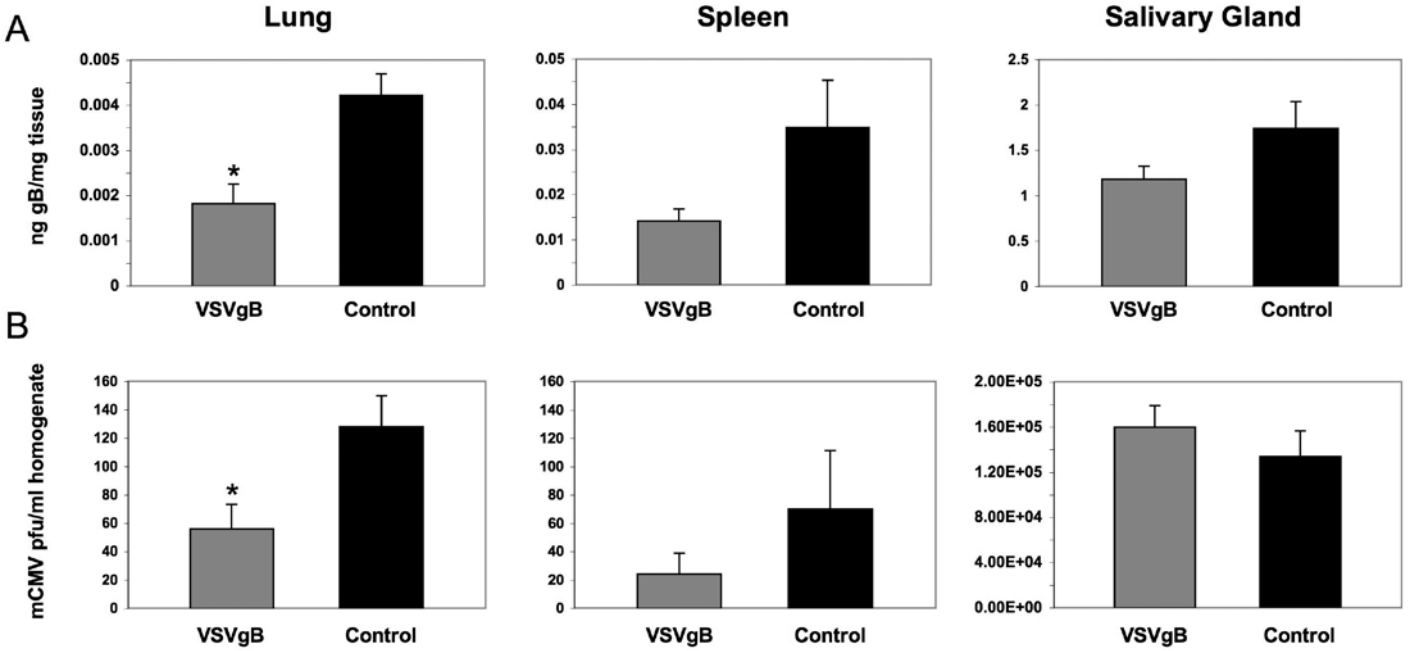

Figure 3. Detection of viral DNA and live virus in rVSV–gB-immunized mice challenged with mCMV. BALB/c mice were immunized intranasally with either 5×10^6 pfu of rVSV–gB (n = 5) or PBS (n = 10) 4 wk before intraperitoneal challenge with 2.25 × 103 pfu of Smith strain mCMV. Ten days after challenge, lung and spleen were harvested and 10% tissue homogenates were assayed by PCR to assess viral load (A) or live virus by plaque assay (B). *, $P < 0.05$ compared with control value.

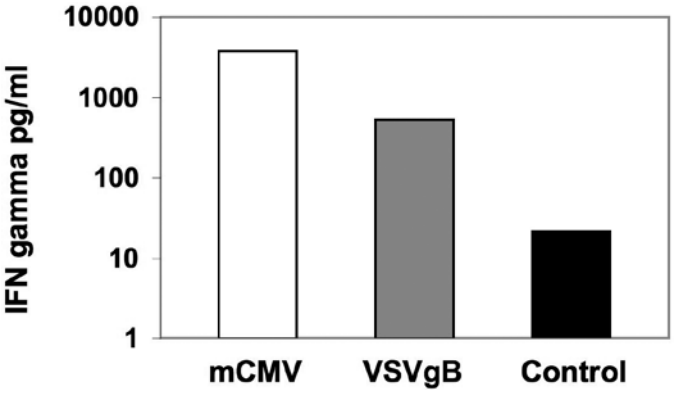

Figure 4. Secretion of IFNγ by rVSV–gB-primed splenocytes in the presence of mCMV antigen. BALB/c mice were immunized intranasally with 5×10^6 pfu rVSV–gB or PBS. As a positive control 1×10^6 pfu Smith strain mCMV was inoculated intraperitoneally. After 8 d, splenocytes were harvested and cultured for 72 h in the presence of inactivated mCMV. Culture supernatants were collected, and the concentration of IFNγ was determined by ELISA.

to lie in its effect in controlling viral dissemination from infected to neighboring cells and the resultant inhibition of viral replication.[33,59] Although neutralizing antibodies are considered an important component of host immunity to mCMV, mechanisms of viral clearance rely upon cellular immunity, with the responsible effector cells varying among infected organ systems.[32,60,62,63]

Intranasal vaccination of mice with rVSV–gB produces a strong cellular immune response, a key component in protection against CMV disease.[66,69] In the present study, primed splenocytes from immunized mice exhibited an increase in IFNγ secretion when cultured with inactivated mCMV. Our intracellular cytokine experiments demonstrated an elevation in IFNγ levels in splenocytes from rVSV–gB-immunized mice which was due, in large part, to an increase in the percentage of activated $CD8^+$ cells. Of interest, the percentage of cells staining positively for both IFNγ and CD8 in splenocytes primed by the vaccine approached that seen by priming with live mCMV. Studies of IFNγ-deficient mice have demonstrated the importance of IFNγ in controlling mCMV infection in the lungs during primary infection.[18] $CD8^+$ cells also play an important role in the control of virus multiplication in the lungs.[59] Recombinant VSVs expressing foreign proteins induce strong antigen-specific $CD8^+$ T-cell responses characterized by target cell lysis and cytokine secretion.[23,24,42,53,55,76] In the present study, vaccination of mice with rVSV–gB resulted in elevated IFNγ levels and an increase in $CD8^+$ T cells, coupled with a protective effect against infection in the lungs. $CD8^+$ T cell proliferation may occur in the absence of helper $CD4^+$ T cells but is dependent on the presence of IFNγ-, IL2-secreting $CD8^+$ T cells.[90] Enhancement of CMV-specific $CD8^+$ T cells capable of effective cytokine production, in particular IFNγ and its activation of genes implicated in antigen presentation, cell adhesion, and chemotaxis may prove beneficial in minimizing CMV reactivation in immunocompromised patients.[7,25]

Intranasal immunization of mice with rVSV–gB successfully reduced mCMV infection in lungs. Both PCR and plaque assays demonstrated statistically significant reductions in live mCMV and viral DNA recovered from lung homogenates. An effective immune response to a primary mCMV infection, resulting in a reduction of viral DNA, likely is associated with lower levels of latent virus and therefore lower reactivation levels. Mean values for splenic PCR and plaque assay results did not achieve statistical significance.

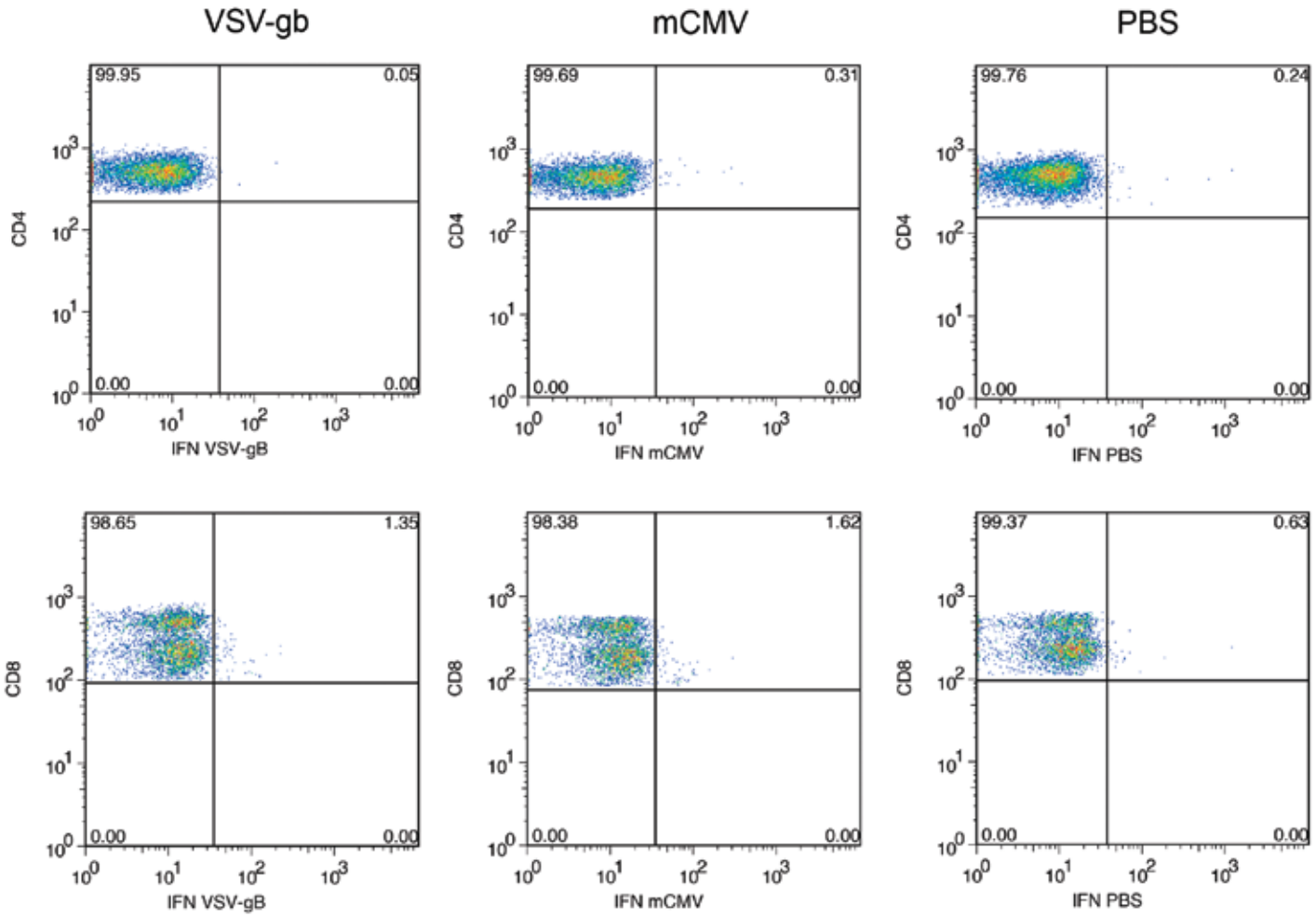

Figure 5. Intracellular IFNγ staining of CD4+ and CD8+ T cells after intranasal immunization with rVSV–gB. BALB/c mice were immunized intranasally with 5×10^6 pfu rVSV–gB or PBS. Positive control mice were inoculated intraperitoneally with 1×10^6 pfu Smith strain mCMV. After 8 d splenocytes were harvested, cultured for 16 h in the presence of inactivated mCMV, and stained with anti-IFNFγ antibodies along with antibodies against CD4 (upper) and CD8 (lower). Numbers in the upper right corner of each flow cytometry plot indicate the percentage of splenocytes double-staining positively for both IFNF and T cell subtypes.

Plaque assay results demonstrated no reduction in the recovery of live mCMV from the salivary glands of rVSV–gB-immunized mice. Murine CMV replication in all central organs and the salivary gland acinar fibroblasts is under the control of $CD8^+$ cells, whereas viral clearance in the salivary gland acinar epithelial cells is resistant to $CD8^+$ control.[40] Studies of mCMV infection in $CD4^+$ deficient mice have demonstrated that this T-cell subset is essential for viral clearance from the salivary glands.[32,33] We also examined whether immunization with rVSV–gB induced a significant increase in IFNγ production by $CD4^+$cells when cultured with inactivated mCMV. The proportion of $CD4^+$ cells producing IFNγ was equal for both control and rVSV–gB groups. Without an enhanced $CD4^+$ response, clearance of virus from the salivary glands remains problematic.

Whether the immune responses elicited by vaccination with rVSV–gB are due in large part to antigen-specific mechanisms rather than nonspecific immunity requires further investigation. Challenge studies examining rVSVs expressing foreign viral proteins have shown that protection can be attributed to antigen specific rather than nonspecific immune responses.[34,47,72,74,79] In the present study, the final readout for the immunofluorescent experiments was antibody binding to cells constitutively expressing mCMV-gB on the cell surface. The results from sera of rVSV-gB- and rVSV-egfp-immunized mice strongly suggest that the responses were not a result of vector-induced immunity but rather were specific to gB. One proposed advantage of rVSVs is their activation of the immune system because of the nature of the viral envelope, particularly the G protein.[43] Infection with VSVs is assumed to activate interferon responses that elicit an adjuvant effect for vaccine-specific responses to gene inserts.[8] Although demonstrated with other rVSVs expressing foreign viral proteins, cellular immunity could not be definitively proven to be antigen-specific in this study. Further development of rVSVs expressing mCMV proteins will include investigations into antigen specificity in both humoral and cellular immune responses.

Mouse models and human clinical trials have demonstrated that immunization can induce host protection against CMV infection. Vaccination with tissue culture attenuated or chemically inactivated CMVs has been successful in inducing humoral and cellular immune responses, protection against disease, and re-

duction of viral titers.[21,41,45,46,51,52] This success likely is the result of a robust immune response to multiple epitopes expressed on the modified virion. At a minimum, a CMV vaccine likely must express gB and the teguement protein pp65 in order to induce neutralizing antibodies and a cytotoxic lymphocyte response. The additions of CMV glycoproteins (H and N), a regulatory protein (IE1), and the teguement protein pp150 would enhance the immune response.[22] Immunization with a trivalent plasmid DNA (IE1, M84, and gB) followed by a formalin inactivated mCMV boost has been successful in providing long term protection against mCMV replication.[45] Developing a rVSV vaccine targeting multiple mCMV epitopes, including gB, could enhance the immunologic response and partial protection already demonstrated by our vaccine.

Optimization of several factors may enhance the ability of a rVSV expressing gB to provide complete protection against mCMV infection. In our construct, the mCMV gB gene was inserted at a downstream site between the genes encoding for the VSV G and L proteins. Recently a novel rVSV vector has allowed the insertion of foreign genes upstream from the gene encoding for the VSV N protein.[55] Insertion at this 3′–most site would allow maximal expression of the gB gene as it is transcribed first and not influenced by transcription attenuation of upstream genes.[31] In addition, upstream insertion may prevent mutations of the foreign gene that lead to rapid elimination of protein expression sometimes encountered with placement between VSV G and L genes.[54,55]

Effective vaccination against CMV will necessitate alteration of host humoral immunity as well as activation of several arms of the cellular immune system. The present studies demonstrate that the insertion of mCMV-gB into rVSV vector systems results in strong immunogenicity and, when administered as a single intranasal dose, provides partial protection against mCMV. Mice immunized with rVSV–gB exhibited a strong, rapid rise in anti-gB titers and, within days after challenge, showed a significant anamnestic response. Therefore boosting a primary immunization with either a heterologous VSV vector or an unrelated viral vector prior to challenge potentially could provide an increase in immunity. Our results demonstrate the value of these systems and that further development of this vaccine could lead to new strategies for preventing CMV in humans.

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