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Evaluating Replication-Defective Vesicular Stomatitis Virus as a Vaccine Vehicle

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We have generated replication-competent (VSV-C/E1/E2) and nonpropagating (VSVΔG-C/E1/E2) vesicular stomatitis virus (VSV) contiguously expressing the structural proteins of hepatitis C virus (HCV; core [C] and glycoproteins E1 and E2) and report on their immunogenicity in murine models. VSV-C/E1/E2 and VSVΔG-C/E1/E2 expressed high levels of HCV C, E1, and E2, which were authentically posttranslationally processed. Both VSV-expressed HCV E1-E2 glycoproteins were found to form noncovalently linked heterodimers and appeared to be correctly folded, as confirmed by coimmunoprecipitation analysis using conformationally sensitive anti-HCV-E2 monoclonal antibodies (MAbs). Intravenous or intraperitoneal immunization of BALB/c mice with VSV-C/E1/E2 or VSVΔG-C/E1/E2 resulted in significant and surprisingly comparable HCV core or E2 antibody responses compared to those of control mice. In addition, both virus types generated HCV C-, E1-, or E2-specific gamma interferon (IFN-γ)-producing $CD8^+$ T cells, as determined by enzyme-linked immunospot (ELISPOT) analysis. Mice immunized with VSVΔG-C/E1/E2 were also protected against the formation of tumors expressing HCV E2 (CT26-hghE2t) and exhibited CT26-hghE2t-specific IFN-γ-producing and E2-specific $CD8^+$ T-cell activity. Finally, recombinant vaccinia virus (vvHCV.S) expressing the HCV structural proteins replicated at significantly lower levels when inoculated into mice immunized with VSV-C/E1/E2 or VSVΔG-C/E1/E2, but not with control viruses. Our data therefore illustrate that potentially safer replication-defective VSV can be successfully engineered to express high levels of antigenically authentic HCV glycoproteins. In addition, this strategy may therefore serve in effective vaccine and immunotherapy-based approaches to the treatment of HCV-related disease.

Hepatitis C virus (HCV), a member of the *Hepacivirus* genus belonging to the *Flaviviridae* family, is the major etiological agent of parentally acquired chronic infection that affects more than 170 million people worldwide (2). Major sources of transmission include infected blood transfusions or intravenous drug abuse, but lower-risk exposures follow vertical, sexual, and occupational exposure to blood (35, 45, 49). Screening measures with improved diagnostic technologies have lowered the risks of contaminated blood transmission, but annual estimates suggest that 100,000 new infections still occur in the United States alone due to the seronegative "window" period of HCV infection (47). Furthermore, infections are often asymptomatic, and 75% of exposed individuals acquire chronic disease that subsequently contributes to potential liver fibrosis, cirrhosis, and possibly hepatocellular carcinoma (36). HCV infection is therefore the leading cause of liver transplantation in the United States, and despite several new HCV-specific or general antiviral drugs in clinical development or trials, approved FDA treatments are still based on expensive interferon (IFN) regimens (74). However, even improved combination IFN therapy strategies using pegylated IFN-ribavarin are successful in only 40 to 50% of patients. Alternative preventative or immunotherapeutic interventions are therefore essential to combat HCV infection.

HCV comprises a 9.5-kb single-stranded positive RNA genome that encodes a single large open reading frame of 3,000 amino acids (aa) translated by an internal ribosome entry site located at the 5′ untranslated region. The polyprotein is cleaved into 10 HCV gene products: three structural proteins, namely the capsid (core [C]) and the envelope glycoproteins (E1 and E2); a small hydrophobic polypeptide P7 ion channel; and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) that are essential for viral replication (8). The cleavage of structural proteins from the polyprotein is mediated by host signal peptidase(s), and the remainder of the polypeptide is cleaved by cointeraction of NS2 and NS3 zinc-dependent proteinase and subsequently the NS3 serine protease. An additional HCV protein (F) of unknown function is proposed by a ribosomal frameshift in the sequence encoding the N-terminal region of the polyprotein (10).

Humoral antibody responses to core, NS3, NS4A/4b, and NS5a are typically detected during serological diagnosis (47). However, a key target for antibody-induced viral neutralization includes the envelope glycoproteins E1 and E2 (14, 22, 24, 61). Surrogate models using soluble E2 binding to cell surface molecules have identified potential cellular receptor candidates such as human TAPA-1 (CD81), scavenger receptor class B type 1, glycosaminoglycans, and, more recently, DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin [CD209]) as well as L-SIGN (DC-SIGNR; liver and lymph node specific [CD209L]) (3, 9, 16, 17, 43, 44, 59, 71). The low-density lipoprotein receptor has also been associated with HCV infection by endocytosis studies on a variety of cell lines (3). However, since HCV exhibits extensive genetic heterogeneity, especially within its glycoproteins, the

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presence of E1/E2 antibodies in sera of chronically infected patients and chimpanzees suggests that the responses are likely limited and isolate restricted. In fact, hypervariable region 1 at the N terminus of E2 is proposed as a target of E2 neutralization, but the virus may generate "immune-escape" hypervariable region 1 variants (46). Nevertheless, studies in chimpanzees and patients favor a strategy combining the induction of cellular immune response with the generation of antibody responses to E1/E2, although generating immune responses to other viral proteins may also be important (14, 15, 27, 31, 75). The role of $CD4^+$ T-helper response is also of significance in HCV infection since the major histocompatibility complex (MHC) class II genotype (HLA DRB1*1101/DQ0301) has been associated with spontaneous clearance of infection (76). Furthermore, strong T-helper 1 (Th1) $CD4^+$ responses appear to protect from chronic infection (69, 70). During chronic infection, however, patients appear to mount weak or strong CD4/CD8 responses that subsequently wane and the effector functions of which appear impaired (29, 75, 78). Hence the development of efficient technologies capable of inducing strong Th1 $CD4^+$ and $CD8^+$ T-cell responses is probably essential for optimal HCV prophylactic or therapeutic vaccine design. Characterization of immune responses in chimpanzees points to the requirement of vaccine candidates that can stimulate vigorous, sustained, and multispecific Th1 intrahepatic $CD8^+$ and $CD4^+$ T-cell responses that appear associated with resolution of acute infection (15, 75).

Recombinant E1/E2 immunization of chimpanzees, the only true nonhuman permissive infectious model, demonstrates that neutralizing antibodies can protect against low doses of homologous HCV challenge but not heterologous virus (14, 37). However, as confirmed in more recent studies, the duration of viremia was significantly shortened following reinfection, and E2 subunit vaccines could reduce liver disease (61). Furthermore, in intravenous drug users, previously infected individuals appear less likely to develop new viremia, suggesting a positive role for immunity against HCV pathology (50). HCV vaccines may therefore reduce the common problem of HCV chronicity and morbidity or mortality associated with HCV-related liver disease. In this endeavor, many approaches to more recent HCV vaccine strategies have attempted to improve immunogenicity to antigens and protective efficacy of subsequent immune responses using chimpanzees and smaller animal models. Examples of such strategies include incorporation of HCV proteins into recombinant viruses, naked DNA vaccines with or without boosting with recombinant proteins to improve cytotoxic T-lymphocyte (CTL) responses, virosome or liposome carriers for immunogenic peptides to enhance their delivery to cells, and use of HCV-like particles to mimic virions (11, 33, 51, 55, 62). Use of the more conserved HCV core proteins among HCV genotypes as an adjuvant for envelope glycoprotein vaccine has similarly proven useful in rhesus macaque studies (60).

Recently, we have described the usefulness of vesicular stomatitis virus (VSV) as a tool for delivering HCV immunity in mice (23). VSV vectors have been shown to exhibit significant promise in vaccine studies against respiratory syncytial virus, human papillomavirus, and AIDS-related diseases (40, 64, 65, 68). More recently, further strategies have been developed to generate safer nonpropagating viruses that lack the VSV glycoprotein (G) that is essential for infectivity (66). Recombinant VSV (rVSV) lacking the G gene (ΔG) can be generated by supplying the G protein in *trans*. Resultant viruses are therefore capable of highly tropic infectivity for one cycle, but progeny viruses are incapable of infecting other cells since they lack the G gene in their genome. Here we report that despite the inability of progeny viruses (ΔG) to undergo recurrent cycles of infection, they induce robust immune responses in vaccine studies compared to their replication-competent counterparts.

MATERIALS AND METHODS

Plasmids and construction of recombinant VSV. The plasmids required for construction of recombinant VSV (pVSV-XN2-Indiana strain, pBl-N, pBl-P, and pBl-L) were provided by John Rose (Yale University) (13, 25, 34, 40). The VSV used was the Indiana strain. For construction of VSV-HCV-C/E1/E2 (VSV-C/E1/E2), the C/E1/E2 region (aa 1 to 746) was cloned into the XhoI and NheI restriction sites of the pVSV-XN2 plasmid (HCV genotype 1b cDNA was obtained from T. Miyamura; accession no. D89815). The C/E1/E2 region was amplified by PCR using primers that added an XhoI restriction site at the 5′ end of the gene sequence and an XbaI restriction site to the 3′ end. To obtain recombinant virus, BHK cells were infected with vaccinia virus (VV) encoding the T7 polymerase (vTF7-3) at a multiplicity of infection (MOI) of 10 for 1 h in Dulbecco's modified Eagle's medium (DMEM)–2.5% fetal bovine serum (FBS). The infected BHK cells were then transfected with pVSV-XN2-C/E1/E2, pBL-N, pBl-P, and pBL-L plasmids according to the Lipofectamine Plus protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). After 3 h, transfection medium was removed and replaced with complete DMEM–10% FBS. After 48 h, cell medium was collected and the dead cells were removed by centrifugation. The medium was then filtered through a 0.2-μm syringe filter to remove residual vaccinia virus, and the final flowthrough was used to infect a fresh dish of BHK cells. Large-scale preparations of virus for mouse studies were made by infecting 10 (150-mm) dishes of BHK cells at an MOI of 0.01 for 24 h. The infection medium was collected, and the virus was ultracentrifuged through a 10% sucrose–phosphate-buffered saline (PBS) cushion at 100,000 rpm. Viral pellets were then resuspended in PBS and stored at −80°C. The generation of the VSV-green fluorescent protein (GFP) construct has been described previously (23).

Replication-defective VSVΔG was generated by inserting a linker encoding the restriction enzyme sites MluI, BsiWi, PmeI, and XhoI into pVSV-XN2 using the MluI and XhoI restriction sites. The MluI restriction site in pVSV-XN2 is located after the transcriptional start signal of the G gene but before the ATG, and the XhoI site is located 3′ of the G gene (42). This caused the entire G gene to be replaced with the multiple cloning site linker, creating the pVSVΔG vector. To construct VSVΔG-C/E1/E2, the core, E1, and E2 region of HCV (as described above) was amplified by PCR with the BsiWi and XhoI restriction sites at the 5′ and 3′ ends, respectively, and inserted into the pVSVΔG plasmid.

The VSVΔG and VSVΔG-C/E1/E2 viruses were made as described above with the following alterations. To construct the virus, a plasmid expressing the VSV G protein (pVSVG) was transfected 24 h before the vTF7-3 infection and again with the pVSVΔG, pBl-N, -P, and -L transfection that follows the vaccinia virus infection. This provides G protein, which is no longer encoded in the parent plasmid, so that infectious virus can be assembled. To grow the virus, BHK cells were transfected with pVSVG 24 h before infection so the resulting virions would have the G protein on the surface and be capable of infection. Any cell that is subsequently infected without previously expressing the G protein will not yield infectious virus.

Growth curves and viral titration. VSV infections were performed (also see below) by infecting cells at the indicated MOI in serum-free medium for 1 h. The inoculum was aspirated and replaced with complete medium containing 10% FBS. Viral titers were determined by conducting 10-fold serial dilutions of each sample and then infecting confluent BHK cells as described above. The cells were then overlaid with 1% low-melting-temperature agarose in complete DMEM. Plaques were then counted 24 h postinfection after being stained with crystal violet; the titers are presented as PFU/ml. Titrations of replication defective VSV were conducted on BHK cells previously transfected with pVSVG (helper cells). One-step growth curve analysis of VSVΔG or VSVΔG-C/E1/E2 was performed in BHK helper cells at an MOI of 10. Culture medium (100 μl) was removed at each indicated time point, and the titer was determined on BHK helper cells as described above.

Recombinant vaccinia virus. vvHCV.s (51) and vv-WR (Western Reserve) (39) were gifts kindly provided by Jake Liang from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). VV stocks were propagated in HeLa cells, and standard vaccinia virus plaque assay was performed in the BSC-1 cell line.

Antibodies. Conformation-dependent mouse anti-E2 monoclonal antibody (MAb) H33 was kindly provided by Jean Dubuisson (Unité Hépatite C, CNRS-UPR2511, Institute de Biologie de Lille Cedex, France), as was the conformation-independent anti-E2 MAb, H52. Conformation-independent MAb to E1, A4, was also provided by J. Dubuisson as a gift from Harry Greenberg (Stanford University). Human anti-E2 MAbs CBH-7 and CBH-8C were obtained from Steven Foung (Stanford University). Polyclonal rabbit anti-E2 was provided by Charles Rice (Rockefeller University). Mouse core MAb was purchased from ViroStat, Portland, Maine.

Cell culture. Huh-7, BHK, HeLa, and BSC-1 cells were propagated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 IU/ml (each) penicillin and streptomycin (DMEM-C). Mouse splenocytes were incubated in RPMI supplemented as described above and also with 0.2 ng/ml mouse interleukin-2 (IL-2) (Endogen, Rockford, IL) and corresponding peptide. Mouse tumor cell line CT26 was maintained as described above, and CT26-hghE2t, which expresses a genotype 1b HCV E2 (aa 384 to 719) protein was grown in DMEM-C and 350 μg/ml G418 selection medium (73).

Radiolabeling HCV glycoproteins. Huh-7 or BHK cells grown in 150-mM dishes were infected with VSV-C/E1/E2 or control VSV-GFP at an MOI of 10 in serum-free DMEM. After 1 h, the cells were washed in PBS and complete growth medium was added, as described previously. Following a 5-h infection period, cells were depleted of methionine and cysteine for 30 min and 600 μCi [^{35}S]Met/Cyst was added. The infection was allowed to proceed for a maximum of 12 h. Cells were then scraped and lysed (0.5% NP40 in 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 20 mM iodoacetamide, and 1 μg of proteinase inhibitors). The lysate was clarified by centrifugation in an Eppendorf centrifuge for 10 min and then precleared with 2.5 μl of normal mouse immunoglobulin G (IgG; Santa Cruz Biotechnology, Santa Cruz, CA) complexed to protein G (Invitrogen, Carlsbad, CA) in immunoprecipitation (IP) buffer (0.2% NP-40 in 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA). HCV E1 and E2 were coimmunoprecipitated with 10 μl (approximately 10 μg) of H33 MAb-protein G with continual shaking at 4°C. After a minimum of 4 h, the immunocomplexes were centrifuged and the pellets were washed and centrifuged several times in IP buffer. For the final wash, the complexes were equally divided into two tubes and the pellet was resuspended in either reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (200 mM Tris, pH 6.8, 0.5% SDS, 10% glycerol, 0.01 g bromophenol blue, 2% β-mercaptoethanol) or nonreducing buffer (SDS sample buffer without β-mercaptoethanol). After boiling in the respective SDS-PAGE sample buffer for 5 min, the complexes were separated on 10% polyacrylamide gel. The gel was treated with NAMP100 Amplify (Amersham Biosciences, Piscataway, NJ) and then dried prior to exposure to autoradiograph film.

Immunoblotting. Infection procedures and immunoprecipitation prior to Western analysis were carried out as described for radiolabeling experiments. Briefly, Huh-7 or BHK cells were infected at an MOI of 10 for 1 h with live VSV-C/E1/E2, VSV-GFP, or nonpropagating VSVΔG-C/E1/E2 or VSVΔG. The inoculum was removed, the cells were washed, and growth medium was added to allow the infection to proceed for 12 h. Cells were then harvested as before, and immunoprecipitations were performed using the panel of MAbs. Immunocomplexes were resuspended in SDS-PAGE sample buffer (with or without β-mercaptoethanol) and then run on 10% polyacyrlamide gel. Proteins were then transferred to nitrocellulose membranes (Hybond-ECL; Amersham) using a vertical slab gel unit apparatus (model SE400; Hoefer Scientific Instruments, San Francisco, CA) and revealed using anti-E2 rabbit polyclonal antibody (diluted 1/1,000) followed by a mouse anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP; diluted 1/5,000; Santa Cruz Biotechnology, CA). Immunoblots were then analyzed by enhanced chemiluminescence detection (ECL; Amersham, Piscataway, NJ) as recommended by the manufacturer. To detect E1, the mouse anti-E1 A4 antibody (diluted 1/1,000) was used followed by goat anti-mouse antibody (1/5,000) conjugated to HRP. Core protein was detected using a mouse anti-core antibody (Virostat, Portland, Maine) diluted 1/500 followed by the HRP-conjugated goat anti-mouse antibody. For detection of E1 and E2 heterodimers, the membranes were incubated in prewarmed stripping buffer (50 mM Tris, 2% SDS, 0.7% β-mercaptoethanol) for 30 min and then extensively washed in PBS–0.05% Tween followed by reincubation with antibody and chemiluminescent detection.

Immunization of mice. Female BALB/c mice (aged 6 to 8 weeks; Jackson Laboratories, Bar Harbor, Maine) were immunized intravenously (i.v.) or by intraperitoneal (i.p.) injection with VSVC/E1/E2, VSVΔG-C/E1/E2, or control virus (VSV-GFP or VSVΔG) at 6×10^6 PFU in PBS or with PBS alone. A 100-μl total volume of PBS-virus or PBS alone was administered twice during a 3-week interval (day 1 and day 14). Bleeding of mice before or after treatment was undertaken using the microhematocrit tube method. The splenocyte isolation procedure is described below. Mice to be challenged with CT26-hghE2t cancer cells (or control CT26) were given a second boost on day 28 and 2 weeks later received cancer cells (as described below). In viral challenge experiments, 1×10^7 recombinant vaccinia virus PFU (vvHCV.S or vv-WR) were administered intraperitoneally 2 weeks following the second boost.

HCV core and E2 ELISA. HCV core (genotype 1a) protein expressed in *Saccharomyces cerevisiae* (ViroStat, Portland, Maine) or full-length E2 protein (genotype 1a) purified following baculovirus expression (ImmunoDiagnostics, Inc., Woburn, MA) was diluted (0.5 μg) in coating buffer (50 mM Na_2HPO_4 [pH 9.6]) and bound overnight at 4°C to Nunc-Immuno MaxiSorp surface plates. Wells were washed several times in PBS–0.05% Tween 20 and blocked with PBS–10% heat-inactivated FBS (HI-FBS) at room temperature (RT). Subsequently, the plates were washed again prior to addition of mouse serum (1:100) or control antibody (anti-core; 1:500; and anti-E2, 1:1,000) in PBS–10% HI-FBS for 2 h at RT. Following the wash steps, goat anti-mouse HRP-conjugated antibody (1:4,000 in PBS–10% HI-FBS) was added to test sera and control wells for 1 h at RT. The wells were then washed, and substrate was added for 30 min in the dark, after which the reaction was stopped using 2 N H_2SO_4. The plates were read using an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm (subtracted from 570 nm).

Peptides. The core (aa 133 to 142), E1 (aa 315 to 322), and E2 (aa 570 to 584) peptides have been described previously (23). HCV (genotype 1b) CTL peptides (Genemed Synthesis, Inc., South San Francisco, CA) (core, LPRRGPRLGV RATR [aa 37 to 50] and RRGPRLGVRATRKT [aa 37 to 52]; E2, SGPSQK IQLV [aa 405 to 414] and PPQANWFGCTWMNSTGFTKT [aa 544 to 563]) and a control human immunodeficiency virus peptide (RIQRGPGRAFVTIGK [aa 308 to 322]) were also selected for additional enzyme-linked immunospot (ELISPOT) analysis (12, 56).

ELISPOT assay. Nunc Maxisorp plates (Nunc) were coated by overnight incubation at room temperature with a 5-μg/ml solution of anti-mouse IFN-γ MAb (BD Biosciences, PharMingen, San Diego, CA). The plates were washed (PBS–0.1% Tween), and wells were blocked with RPMI–5% FCS for 1 h at room temperature. Splenectomies were performed on mice sacrificed 21 days postimmunization, and the spleens were removed to a petri dish containing cold sterile PBS. After homogenization, splenocytes were transferred to 50-ml conical tubes and centrifuged at 1,800 rpm for 5 min. The pellets were resuspended in PBS followed by cold sterile water, the tubes were inverted for several seconds, 10× PBS was added, and the suspensions were vortexed prior to centrifugation. Pellets were resuspended in 30 ml of RPMI 1640 (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate, 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 10% heat-inactivated FCS (HyClone, Logan, UT), and the cell suspension was poured through a sterile cell container with a 40-μm pore diameter (Falcon, BD-Biosciences, Bedford, MA). Harvested cells (from 1×10^6 to 2.5×10^5) from each mouse set were seeded in triplicate into 96-well plates precoated with IFN-γ MAb. Cells were stimulated with 0.2 ng/μl of mouse IL-2 (Endogen, Rockford, IL) and pulsed with 10 μg/ml of core, E1, or E2 peptide (described above) for at least 36 h at 37°C. In addition, cells were plated as mock (nonpulsed) controls for each mouse group. For E2-specific CTL responses in CT26-hghE2t tumor-challenged mice, splenocytes were restimulated with mitomycin C-treated (25 μg/ml) cancer cells for 1 week at a ratio of 10:1 splenocytes to CT26-hghE2t cancer cells. The cell medium was removed, and plates were washed extensively with PBS and PBS–0.1% Tween followed by addition of 0.5 μg/ml of anti-IFN-γ MAb (BD Biosciences, PharMingen, San Diego, CA) to each well. Following a 90-min incubation at room temperature and additional washes in PBS–0.1% Tween and PBS alone, 100 μl of 0.2 μg/ml streptavidin-alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) was added to each well. The wells were incubated for 1 h at room temperature and washed, and 3% type 1 low-electroendosmosis agarose (Sigma, St. Louis, MO) and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate in AMP buffer (75 mg $MgCl_2$ hexahydrate, 50 μl Triton X-405, 500 mg NaN_3, 47.9 ml 2-amino-2-methyl-1-propanol [Sigma, St. Louis, MO], pH 10.25) were added to wells. The developed spots were then counted with the aid of an inverted microscope the next day. Any background ELISPOTS from mock wells were subtracted from the experimental groups; the results are presented as mean ELISPOTS per million cells ± standard deviation for each group.

Lymphoproliferative assay. Splenectomies were performed as described above for ELISPOT analysis. Splenocytes were plated in triplicate at dilutions of 1×10^6 to 2.5×10^5 cells in RPMI–10% FBS and 100 IU/ml (each) penicillin and streptomycin in a 96-well format. The cells were stimulated with HCV (genotype

1a) core protein (Virostat, Portland, Maine) at 0.1- and 0.01-μg/ml concentrations, and control cells were stimulated with medium alone for 5 days. The cells were pulsed with 1 μCi of [^{3}H]thymidine per well for at-least 18 h prior to the 5-day incubation. The cells were harvested (Filter maid harvester; Perkin-Elmer, Fremont, CA), and radiation counts were subsequently analyzed.

Tumor challenge experiments. For the CT26-hghE2t and CT26 cancer cell challenge (73), mice were prevaccinated with replication-defective VSVΔG-C/E1/E2 or VSV-ΔG (eight mice per group). A third PBS group (eight mice) was also included. Two weeks after the second boost, 2×10^6 CT26-hghE2t or CT26 cells were injected subcutaneously into each mouse group. The cancer growth was measured every 3 days, and mice were sacrificed when cancers reached approximately 20 mm in diameter or if cancers became highly necrotized.

Viral challenge of mice. Female BALB/c mice (six per group) immunized (days 1, 14, and 28) with replication-defective VSVΔG-C/E1/E2 or VSV-ΔG were challenged with 1×10^7 PFU of vvHCV.s (51) or control wild-type vv-WR (39) by intraperitoneal injection 2 weeks after the last immunization. In addition, no immunized mice were infected with vvHCV.S or vv-WR as controls. Mice (six per group) immunized with replication-competent VSV-C/E1/E2 or VSV-GFP were also challenged as described above. All mice were sacrificed 5 days after challenge, and their ovaries were harvested. Following freeze-thaw and homogenization, vaccinia virus titers were determined in BSC-1 cells by plaque assay.

RESULTS

Viability of VSVΔG-C/E1/E2 and expression of HCV core E1/E2. We have previously demonstrated that replication-competent VSV can be genetically engineered to express HCV structural antigens and that this strategy can be used to generate immune responses in BALB/c mice (23). To evaluate the use of a nonpropagating form of rVSV vector in HCV vaccine strategies, the entire G gene from the parent pVSV-XN2 encoding recombinant VSV was deleted (as described in Materials and Methods) to create the pVSVΔG vector. The entire HCV structural region containing core, E1, and E2 of HCV genotype 1b (aa 1 to 746) was subsequently cloned into pVSVΔG (Fig. 1A). Nonpropagating VSVΔG-C/E1/E2 or control VSVΔG virus was recovered by expression of VSV N, P, and L genes in BHK cells harboring expression of VSV G. Infectious VSVΔG-C/E1/E2 was plaque purified, and its growth properties were compared to those of VSVΔG (Fig. 1B). Virus titers peaked exponentially between 4 and 12 h of infection and then reached a plateau 24 h postinfection at 5×10^6 and 1×10^8 PFU/ml for VSVΔG-C/E1/E2 and VSVΔG, respectively. VSVΔG titers were 1.5 logs higher than those of VSVΔG-C/E1/E2, possibly due to slight attenuation in the latter virus due to the addition of longer heterologous gene products (6). However, initial VSVΔG-C/E1/E2 titers were generally comparable to those previously reported for the replication-competent VSV-C/E1/E2, VSV-GFP, and parental VSV-XN2 (23). To confirm HCV gene expression, BHK cells were infected with VSVΔG-C/E1/E2 or VSVΔG at an MOI of 10 for approximately 24 h, at which point a cytopathic effect was clearly visible (data not shown). The cells were harvested and lysed as described previously, and SDS-PAGE analysis followed by immunodetection of target proteins was performed (Fig. 1C). This study confirmed that both VSVΔG and VSVΔG-C/E1/E2 lacked the VSV G protein. A trace amount of residual G protein was detected, however, plausibly from the original infection of cells. Subsequently, significant HCV C/E1/E2 expression was confirmed in VSVΔG-C/E1/E2-infected BHK cells following immunoblot analysis. Analysis of equal amounts of total cell lysates demonstrated a similar level of correctly sized proteolytically processed HCV C, E1 (35 kDa), and E2 (62 to 68 kDa) protein expression (Fig. 1C). VSVΔG-C/E1/E2 expression followed by Western blotting confirmed detection of the immature (22 kDa) and mature (19 kDa) HCV core products that have been described in other studies (38, 80). These data would suggest that nonpropagating VSVΔG-C/E1/E2 expresses heterologous HCV proteins to levels similar to those of its replication-competent counterpart.

VSV-expressed HCV glycoproteins form noncovalently linked heterodimers and recognize conformationally sensitive MAbs. We have previously reported that VSV-expressed E1 and E2 coimmunoprecipitate as heterodimers in infected cell lysates (23). Here we further evaluated HCV glycoprotein authenticity using conformationally sensitive and nonconformationally dependent MAbs. To accomplish this, we infected Huh-7 cells with VSV-C/E1/E2 or VSV-GFP (as described in Materials and Methods) and carried out immunoprecipitation analysis on cell lysates using conformationally sensitive anti-E2 antibody H33 or conformationally independent anti-E2 antibody H52. The immunoprecipitated products were divided equally, analyzed under reducing or nonreducing SDS-PAGE conditions, and subjected to immunoanalysis using a polyclonal rabbit anti-E2 antibody. This study confirmed that VSV-expressed E2 products interacted with conformationally sensitive anti-E2 H33 and nonconformationally dependent anti-E2 H52 antibodies, appearing as the expected ~68-kDa product under reducing conditions (Fig. 2A). The specificity of VSV-expressed E2 binding to the anti-E2 H33 or anti-E2 H52 was emphasized by the fact that these antibodies did not react to proteins from VSV-GFP-infected cells following similar treatment. Furthermore, mouse IgG did not precipitate E2 from VSV-C/E1/E2-infected lysates, as expected (Fig. 2A). Very small amounts of more highly reactive bands indicative of uncleaved E1/E2 and C/E1/E2 were also evident but were not observed in the control lanes. Previous studies have shown that following HCV glycoprotein expression, disulfide-linked E1/E2 products can exist as unproductive, misfolded protein products that appear as large-molecular-weight aggregates under nonreducing SDS-PAGE (18, 20, 21, 57). However, functional noncovalently linked E1/E2 oligomers appear at their respective sizes of approximately ~35 kDa and ~68 kDa when examined under similar conditions (18, 53, 58). VSV-expressed HCV glycoproteins were therefore immunoprecipitated with anti-E2 H33 and anti-E2 H52 and examined under nonreducing conditions. Our data revealed that anti-E2 H33 bound strongly to E2 of the expected size (~68 kDa), indicating that a significant amount of VSV-expressed E2 product was indeed conformationally authentic (Fig. 2B). In contrast, a weaker signal was seen using the nonconformationally sensitive anti-E2 H52. Since for each IP, equal amounts of lysate were used and equivalent volumes of eluted product were analyzed by SDS-PAGE, the difference in signal was unlikely due to variation in preparation or loading. Plausibly, the anti-E2 H52 antibody may exhibit some capacity to bind both conformationally authentic E2, as evidenced by the presence of some ~68-kDa E2 protein. Misfolded E2 aggregates, in contrast, would appear as higher-molecular-weight products when examined under nonreducing SDS-PAGE conditions or become lodged in loading wells (Fig. 2B). Collectively, our data indicate that correctly sized, conformationally authentic VSV-expressed E2 products can be observed following nonreducing SDS-PAGE analysis of anti-E2 H33 immunoprecipitates.

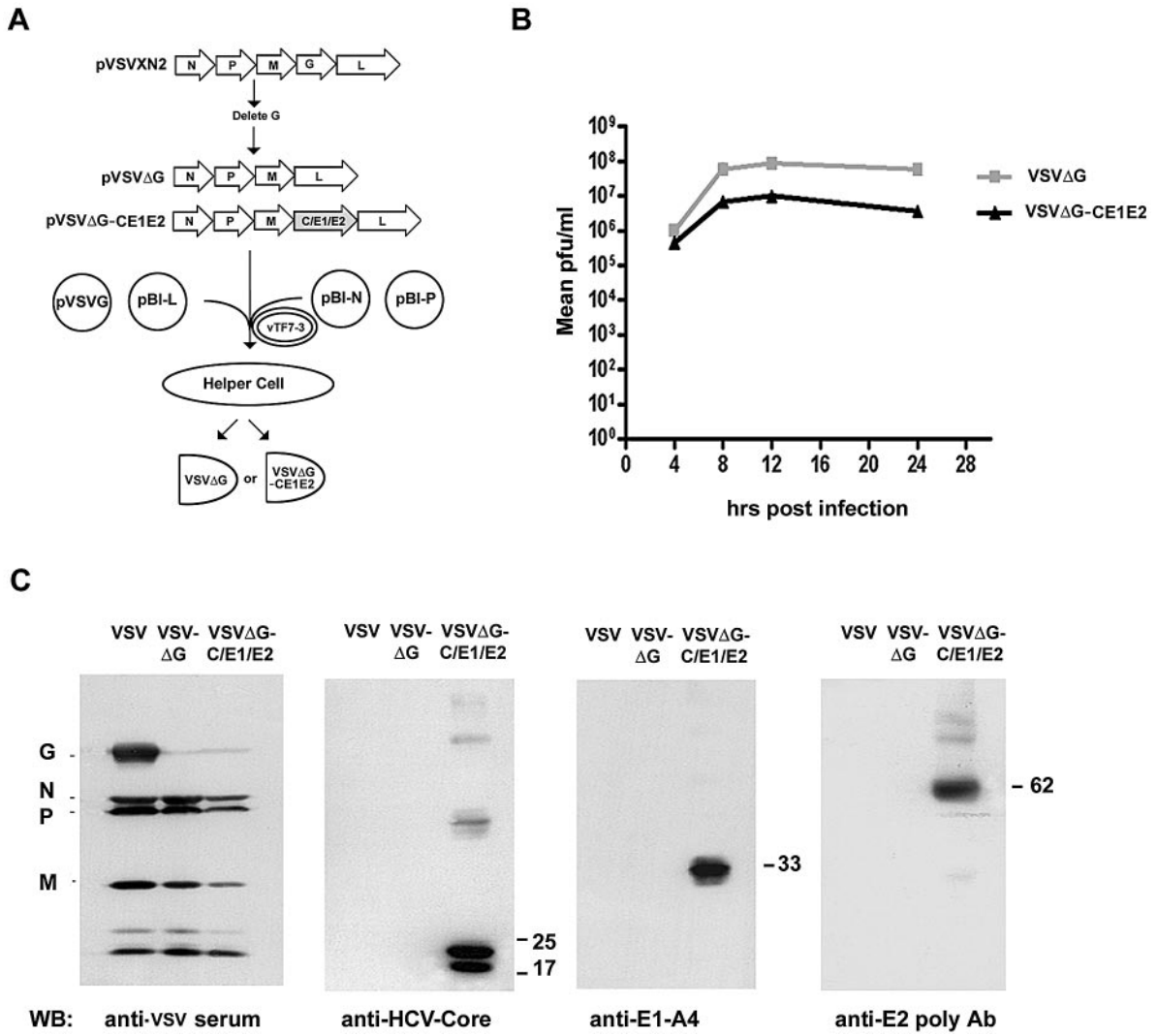

FIG. 1. Development and characterization of a nonpropagating VSV vector expressing hepatitis C virus structural proteins (VSVΔG-C/E1/E2). (A) Construction of nonpropagating rVSV. The complete VSV glycoprotein (G) was deleted from the parent pVSV-XN2 plasmid and replaced with a multiple cloning site linker to create the pVSVΔG vector. The contiguous HCV C/E1/E2 genes were amplified by PCR and inserted into the multiple cloning site in pVSVΔG between the matrix (M) and polymerase (L) genes of VSV-XN2. The VSVΔG or VSVΔG-C/E1/E2 viruses were made by transfecting a pVSVΔG plasmid encoding VSV G into BHK cells. Vaccinia virus encoding the T7 polymerase (vTF7-3) was used to infect these "helper" BHK cells 24 h later (MOI of 10) followed by transfection with pVSVΔG or pVSVΔG-C/E1/E2 pBl-N, -P, and -L. The cells expressing VSV G mediate incorporation of G protein into VSVΔG or VSVΔG-C/E1/E2 virions that can subsequently infect cells. However, since VSV G is no longer encoded in the genome, the virus will be unable to produce infectious virions in cells not expressing the G protein and is therefore replication defective. (B) Growth curve analysis of VSVΔG-C/E1/E1 and VSVΔG. A one-step growth curve analysis was performed in VSV G-expressing "helper" BHK cells. (C) Expression of VSV and HCV proteins in nonpropagating rVSV. BHK cells were infected with wild-type VSV (MOI of 10 for 15 h) or nonpropagating VSVΔG or VSVΔG-C/E1/E2 (MOI of 10 for 24 h), and cell lysates were subjected to Western blot (WB) analysis using mouse VSV-antiserum and the anti-HCV antibodies shown. poly Ab, polyclonal antibody.

As mentioned above, functional HCV glycoproteins are proposed to exist as noncovalently linked E1/E2 heterodimers. We therefore evaluated whether VSV-expressed E1 and E2 exhibited these properties. Accordingly, equal amounts of radiolabeled lysates from VSV-C/E1/E2- or VSV-GFP-infected or uninfected (mock) Huh-7 cells were immunoprecipitated with conformationally sensitive H33 antibody or control IgG. Eluted products were analyzed by nonreducing SDS-PAGE and subsequently exposed to X-ray film. These data indicated that E1/E2 products were clearly apparent, confirming that noncovalently linked and correctly folded E1/E2 heterodimers can be produced by VSV expression (Fig. 2C).

To further confirm the authenticity of our E1/E2 products, we next utilized conformation-dependent human anti-E2 CBH-7 and CBH-8 MAbs (hMAbs) (32). Huh-7 cells were thus infected with VSV-C/E1/E2 (or VSV-GFP), and lysates were subjected to coimmunoprecipitation analysis as described above. CBH-7- or CBH-8-precipitated E2 glycoproteins were confirmed by immunoblotting using rabbit anti-E2 polyclonal antibody under reducing or nonreducing SDS-PAGE condi-

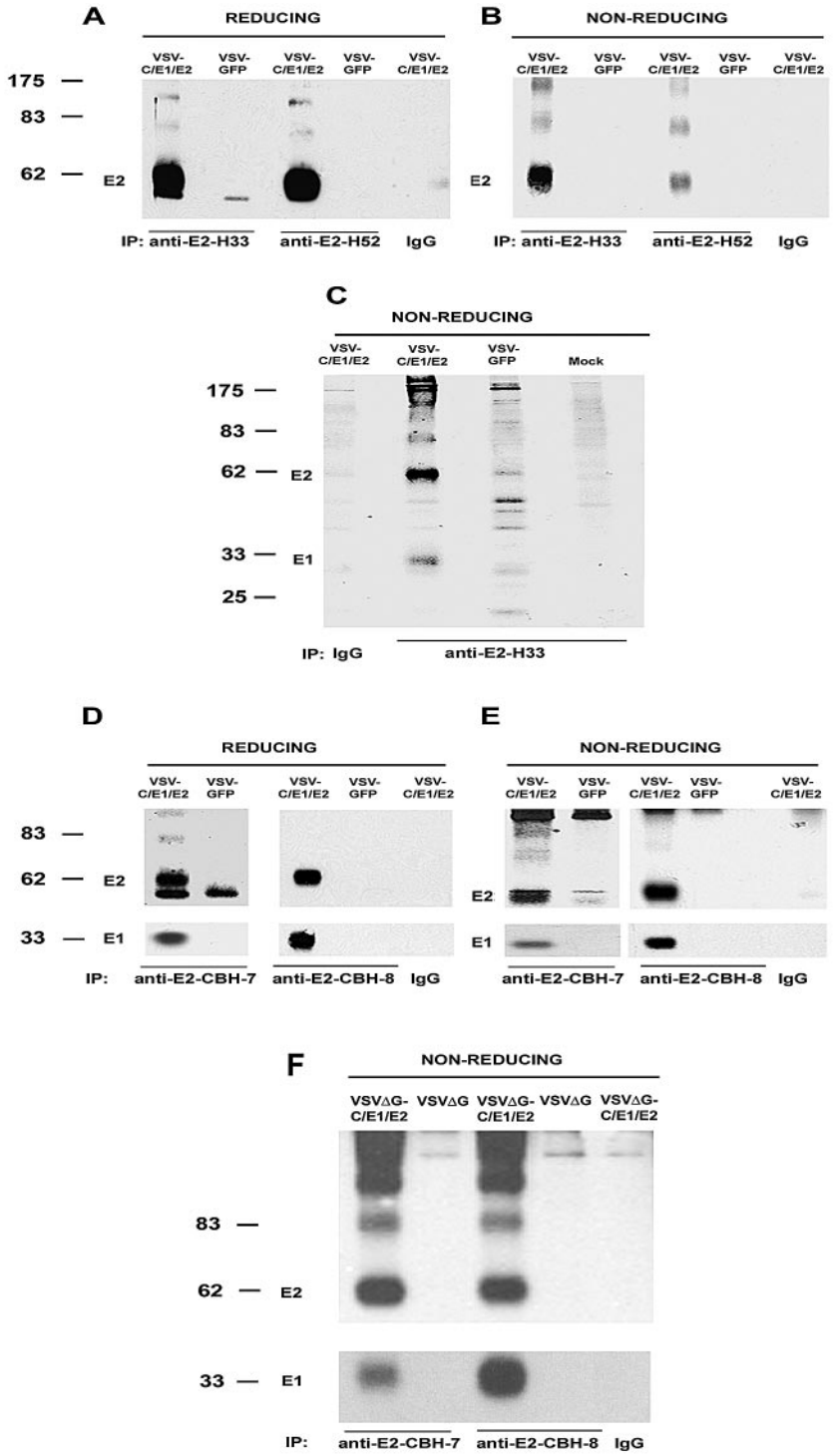

FIG. 2. Characterization of E1 and E2 glycoproteins expressed by VSV using mouse or human conformation-dependent MAbs. Huh-7 cells were infected (MOI of 10 for 12 h) with VSV-C/E1/E2 or VSV-GFP, and the cell lysates were subjected to IP using anti-E2 H33 conformation-dependent or anti-E2 H52 conformation-independent MAb (as detailed in Materials and Methods). The immunoprecipitated protein G complexes were then subjected to reducing SDS-PAGE (A) analysis or nonreducing SDS-PAGE conditions (B) and detected using an anti-E2 polyclonal antibody following the Western blotting procedure. (C) Radiolabeled noncovalently linked E1/E2 heterodimers. Huh-7 cells were infected (MOI of 10) for 5 h with VSV-C/E1/E2 or VSV-GFP. Cells were depleted of Met and Cys before addition of 600 μCi [^{35}S]Met/Cys. Cells were harvested, and lysates were subjected to IP (detailed in Materials and Methods) using the anti-E2 H33 conformationally sensitive MAb. The immune complexes were subjected to nonreducing SDS-PAGE and subsequently to autoradiography. E1/E2 heterodimers bind human conformation-dependent antibodies, as shown under reducing (D) and nonreducing (E) SDS-PAGE conditions. Huh-7 cells were infected as described above, and lysates were investigated using an IP procedure with CBH-7 and CBH-8 hMAbs followed by Western detection with anti-E2 polyclonal antibody (upper panels). The blots were then stripped and reprobed with anti-E1 A4 MAb (lower panels). Huh-7 cells were also infected with VSVΔG-C/E1/E2 or VSVΔG (MOI of 10 for 12 h), and the lysates were subjected to IP as described above using CBH-7 or CBH-8 hMAB followed by SDS-PAGE and Western detection with anti-E2 polyclonal antibody (F, upper panels). The blots were stripped and reprobed with anti-E1 A4 MAb (lower panels).

tions (Fig. 2D and E, upper panels). To detect E1, immunoblots were reprobed with anti-E1 A4. Our findings illustrate that VSV-expressed E1 could indeed be coimmunoprecipitated using anti-E2 CBH-7 or CBH-8 under reducing or nonreducing conditions, presumably since it is appropriately bound to E2 (Fig. 2D and E, lower panels).

To examine the authenticity of VSVΔG-C/E1/E2-expressed E1/E2 products, Huh-7 cells were infected with VSVΔG-C/E1/E2 or VSVΔG at an MOI of 10 for 12 h and the lysates were subjected to coimmunoprecipitation analysis as described above. CBH-7- or CBH-8-precipitated E2 glycoproteins from VSVΔG-C/E1/E2-infected lysates were confirmed by immunoblotting using rabbit anti-E2 polyclonal antibody under nonreducing SDS-PAGE conditions (Fig. 2F, upper panels). The blots were then reprobed with anti-E1 A4 to demonstrate the presence of noncovalently bound E1 (Fig. 2F, lower panel). Collectively, our data illustrate that VSV-expressed HCV E1 and E2 can for the majority form noncovalently linked heterodimers as determined by antibodies generated to native HCV envelope glycoproteins (32). Thus, the VSV-C/E1/E2 and VSVΔG-C/E1/E2 system appears capable of efficiently expressing HCV structural proteins, including E1/E2, a proportional amount of which form correctly processed heterodimers.

Generation of humoral immune responses by VSVΔG-C/E1/E2. Following generation of nonpropagating VSVΔG-C/E1E2 and evaluation of VSV-expressed E1/E2 products with conformationally sensitive MAbs, we next evaluated the potential of VSVΔG-C/E1/E2 as a vector to generate anti-HCV immune responses and compared the responses to those of the replication-competent (VSV-C/E1/E2) virus counterpart. First, immunoblot analysis confirmed that the replication-defective VSVΔG-C/E1E2 expressed HCV core, E1, and E2 at similar levels in BHK cells (MOI of 10; Fig. 3A to C). Subsequently, BALB/c mice were injected twice (day 1 and day 14) with 6×10^6 PFU of replication-competent VSV-C/E1/E2, VSV-GFP, or nonpropagating VSVΔG-C/E1/E2 or VSVΔG by either i.p. or i.v. administration. Control mice were injected with an equal volume (0.1 ml) of PBS. The serum of immunized mice was analyzed for antibody to HCV core or E2 by ELISA at day 0 and day 21 postinoculation. These data revealed that only mice injected with VSV-C/E1/E2 or VSVΔG-C/E1/E2 exhibited potent and highly specific anti-HCV core antibodies following i.p. or i.v. immunization (Fig. 3D, E, F, and G). No significant cross-reacting antibodies to HCV core protein were detected in mouse serum isolated from control or VSVΔG-immunized mice. However, i.p. routes of administration appeared to result in stronger antibody responses to the HCV core. Our data confirm that nonpropagating VSVΔG-C/E1/E2 can be used to express similarly high levels of HCV antigens compared to its replication counterpart, VSV-C/E1/E2, and to elicit robust anti-HCV core antibodies in mice models.

Animals inoculated with either VSV-C/E1/E2 or VSVΔG-C/E1/E2 (i.p. or i.v.) also mediated clear anti-HCV E2 responses following two immunizations as described above (Fig. 4). Although the E2 glycoprotein is highly variable between subtypes and genotypes (46), our data demonstrate that VSV-induced HCV E2 (genotype 1b) antibodies can react with a genotype 1a antigen. The level of anti-E2 response was similar using either VSV-C/E1/E2 or VSVΔG-C/E1/E2 via the i.v. route (Fig. 4C and D), but VSVΔG-C/E1/E2 generated marginally more anti-E2 antibody than VSV-C/E1/E2 via the i.p. route of administration, for reasons that remain unclear (Fig. 4A and B). We may have detected even stronger anti-HCV E2 responses in VSV-C/E1/E2 or VSVΔG-C/E1/E2 mouse sera in the presence of an HCV 1b E2 antigen in our ELISA. Nevertheless, the data presented clearly illustrate the usefulness of nonpropagating VSV expressing HCV structural antigens in generating anti-HCV core and E2 antibody responses in mice. In addition, intraperitoneal administration of recombinant VSV appears to deliver marginally better humoral immunity than intravenous injection.

Since our vaccine regimen appeared to stimulate potent humoral responses, we next examined the profile of anti-HCV IgG isotypes. Following activation, B-cell class switching and the IgG subtypes are influenced by cytokine profiles generated by the Th cell response. Specifically, IL-4 stimulation leads to IgG1, while IgG2a (and sometimes IgG3) isotypes are responsive to IFN-γ, whereas IgG2b switching requires transforming growth factor β (72). Anti-HCV core-positive sera from three mice were therefore tested in an ELISA format, and isotype-specific secondary antibodies were used to detect immunoglobulin (IgG) subtypes. Previous studies of patients have shown that high levels of IgG2a are typical of Th1 responses and correlate with HCV clearance (77). Our findings in BALB/c animals illustrated high levels of both IgG1 and IgG2a anti-HCV core responses following i.p. immunization with VSVΔG-C/E1/E2 (Fig. 5). Although potent IgG1 responses are also shown and are typically indicative of Th2-type responses, the high levels of IgG2a indicate that VSVΔG-C/E1/E2 immunization of mice can stimulate antibody subtypes indicative of Th1-type responses. Collectively, our data would indicate that VSVΔG-C/E1/E2 is able to elicit robust antibody responses to all of the structural proteins of HCV.

Generation of cell-mediated immune responses by VSVΔG-C/E1/E2. Next we analyzed cellular immune responses associated with our immunization model. Specifically, we were interested in evaluating whether our vaccine regimens could generate Th1-type CTL responses, as demonstrated by IFN-γ release by $CD8^+$ T cells. These T-cell subsets have been shown to play an important role in HCV infection (30). Accordingly, splenectomies were performed on day 21 in mice that had been immunized with either VSV-C/E1/E2 or VSVΔG-C/E1/E2 (i.p. or i.v.). Splenocytes from mice were pulsed with 10 μg/ml of genotype 1b core, E1, or E2 peptide with IL-2 and analyzed for IFN-γ production by ELISPOT assay (Fig. 6). Our results indicate that mice immunized with either VSV-C/E1/E2 or VSVΔG-C/E1/E2 generated statistically significant ($P = <0.05$) CTL activity against a panel of HCV structural peptides as compared with control viruses. We found that, in general, higher CTL responses to HCV C, E1, and E2 were observed in VSVΔG-C/E1/E2-immunized mice than in VSVC/E1/E2-immunized mice (the exception being E1 following i.v. injection of VSV-C/E1/E2). Interestingly, the removal of the VSV G protein appeared to modulate a more responsive CTL response to HCV antigens and also a general increase in nonspecific CTL immunity following i.p. injection, for reasons that are yet unclear (Fig. 6B and E). These results indicate that the VSVΔG-C/E1/E2 vaccine strategy can stimulate CTL responses to HCV C, E1, and E2. Furthermore, enhanced CTL

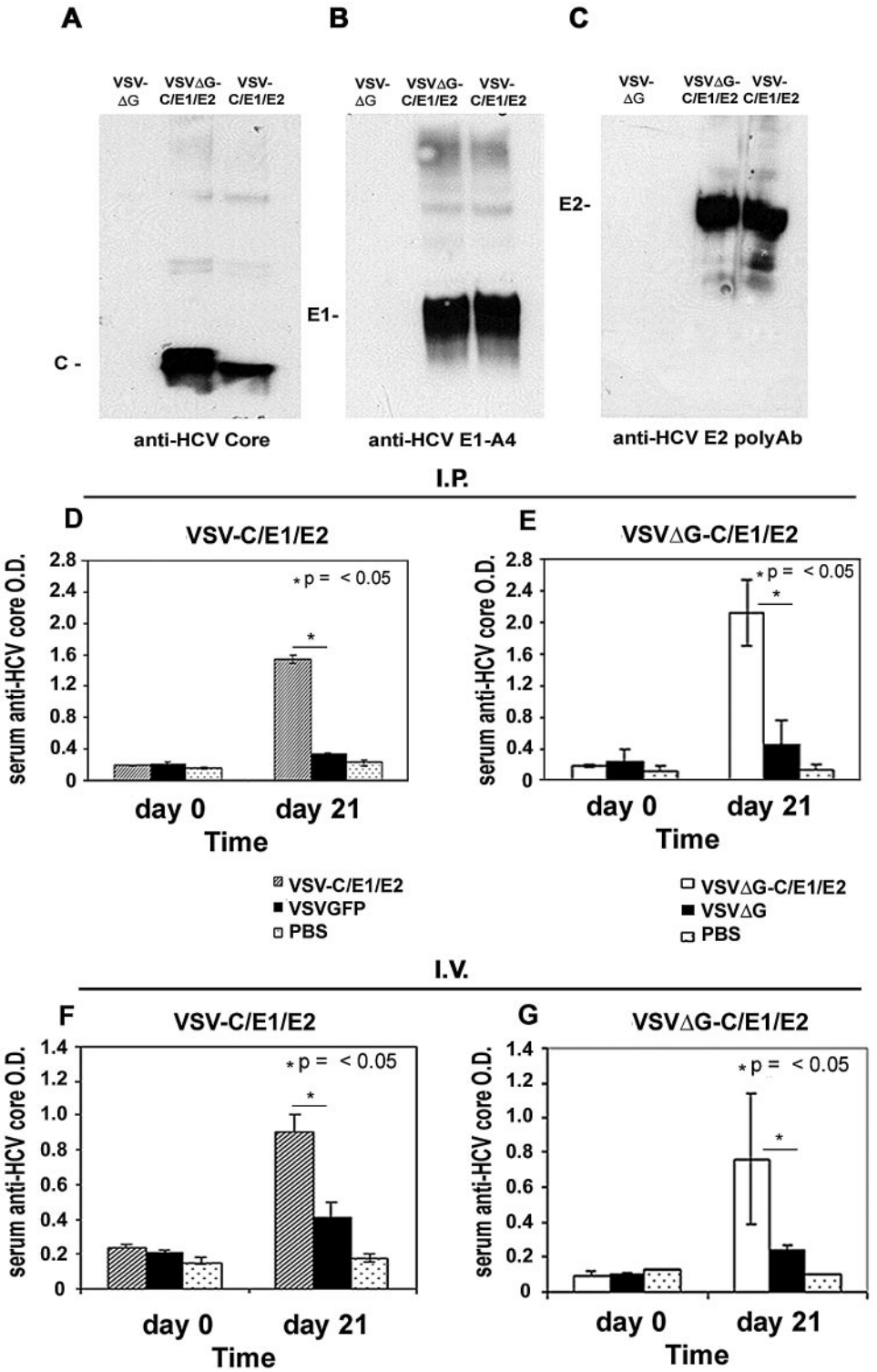

FIG. 3. Comparison of levels of HCV antigen expression between VSVΔG-C/E1/E2 and VSV-C/E1/E2 and antibody responses to HCV core in immunized mice. BHK cells were infected with nonpropagating (VSVΔG or VSVΔG-C/E1/E2) or replication-competent VSV-C/E1/E2 at an MOI of 10 overnight. Harvested cell lysates were subjected to Western analysis with anti-HCV core (A), anti-HCV E1 A4 (B), or anti-E2 polyclonal antibody (polyAb) (C). The same concentration of total protein (20 μg) was used for the Western procedure. Six-week-old BALB/c mice (three mice per group) were vaccinated (day 1 and day 14) with 6×10^6 PFU of VSV-C/E1/E2 or VSV-GFP (or 0.1 ml of PBS only) in parallel experiments with VSVΔG-C/E1/E2 or VSVΔG by the i.p. route (D and E) or i.v. injection (F and G). Serum from all mice was extracted on day 0 and day 21 and tested (in duplicate for each mouse) for anti-HCV core antibody using an ELISA (described in Materials and Methods). The results are presented as mean serum anti-HCV core optical density (O.D.) at 450 nm for each group, and error bars represent standard deviations within each group. Student's *t* test analysis was also performed for comparisons between the virus groups shown, and significant data are illustrated as $P = <0.05$.

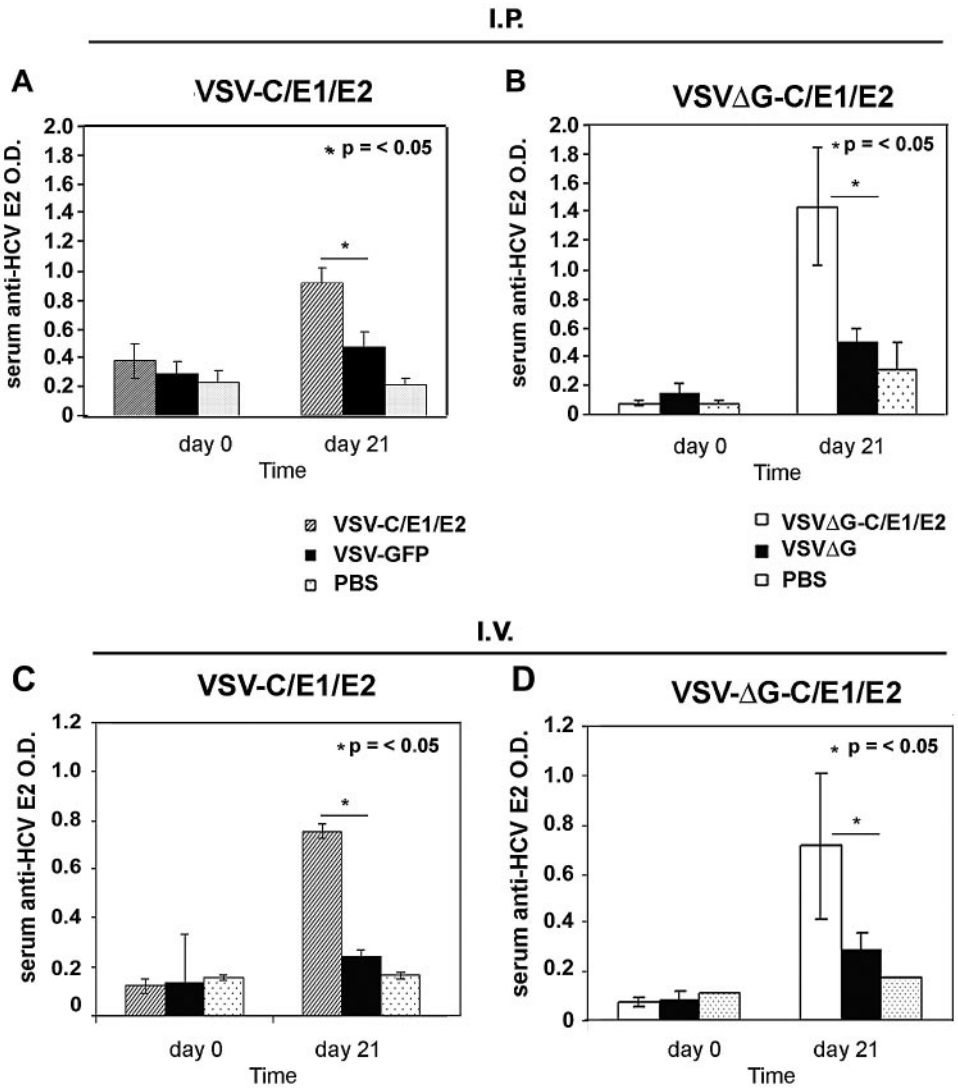

FIG. 4. Comparison of anti-HCV E2 antibody responses stimulated by intraperitoneal or intravenous injection of VSV-C/E1/E2 or VSVΔG-C/E1/E2. Sera from mice tested for anti-HCV core reactivity were used to investigate anti-HCV E2 antibody responses using an HCV E2-specific ELISA (Materials and Methods). Antibody responses to E2 were compared between replication-competent and nonpropagating viruses following i.p. injection (A and B, respectively) and i.v. administration (C and D). Responses are shown as means ± standard deviation of three mice tested in duplicate per group. Student's *t* test analysis was also performed on the data between virus groups, and significant results are shown ($P = <0.05$). O.D., optical density.

responses are achieved with nonpropagating VSVΔG-C/E1/E2 compared to replication-competent VSV-C/E1/E2.

We have previously described lymphoproliferative responses to HCV proteins in VSV-C/E1/E2-immunized mice (23). Here we further demonstrate lymphoproliferative responses in VSVΔG-C/E1/E2 intraperitoneally immunized animals following stimulation of splenocytes with the HCV core protein (Fig. 6F). Although we did not directly study $CD4^+$ responses in this study, the splenocyte proliferation observed will represent both CD8 and CD4 cells following MHC I and MHC II class presentation. However, since CD8 cells are dependent upon the proliferation of CD4 cells as they require an exogenous source of IL-2 to initiate proliferation (28), the total lymphoproliferative responses observed should include HCV core-specific $CD4^+$ T-cell subsets induced by VSVΔG-C/E/1/E2. In addition, our notion that CD4 T cells are induced in our system is corroborated by the induction of strong IgG antibody responses (Fig. 3, 4, and 5). The characterization of these total lymphoproliferative responses is warranted in further studies. However, here we show that lymphoproliferative responses were only observed in VSVΔG-C/E1/E2-immunized mice and not using control viruses. Overall, our data suggest that VSVΔG-C/E1/E2 can induce IFN-γ CTL activity to multiple $CD8^+$ epitopes in HCV structural antigens and total lymphoproliferative responses to HCV core antigen. Our approach may therefore offer a potentially useful and safer approach to delivering HCV immunity using VSV.

Immunization with VSVΔG-C/E1/E2 can inhibit the growth of tumors expressing HCV E2. To evaluate whether the immune responses generated from our VSVΔG-C/E1/E2 could

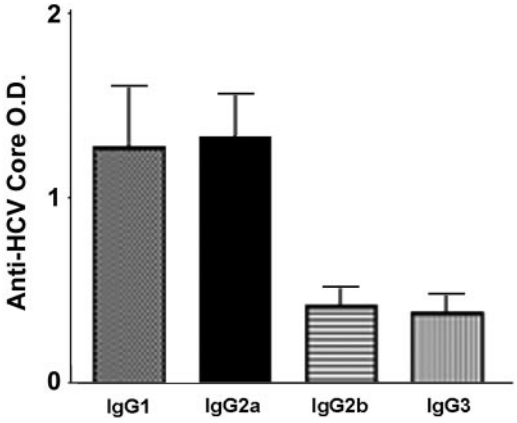

FIG. 5. Analysis of T-helper-cell responses by examining IgG class-switching profiles by B cells. HCV core antibody-positive sera (day 21) from three mice vaccinated with VSVΔG-C/E1/E2 were tested (in duplicate) in the core HCV ELISA and subjected to the isotype-specific anti-mouse secondary antibodies shown. Results are presented as mean anti-HCV core optical density (O.D.) at 450 nm ± standard deviation for each isotype-specific antibody.

be protective against tumor cells expressing HCV E2 protein, we utilized a mouse colon cancer model expressing HCV-E2 (CT26-hghE2t) and looked for protection against malignant disease in immunized BALB/c mice (73). Eight mice per group were intravenously injected with 6×10^6 PFU of VSVΔG-C/E1/E2, VSVΔG, or PBS alone (three inoculations, days 1, 14, and 28). Two weeks following the second boost, 2×10^6 control CT26 or CT26-hghE2t cells in log phase were subcutaneously injected into immunized mice or a control non-immunized group. The number of mice that presented with palpatable tumors was examined every third day, and measurement of average tumor diameter as well as calculation of tumor volume (according to the formula $0.5 \times \text{length} \times \text{width}^2$ (52) began on the 8th day postinsertion of tumor cells (Fig. 7). All control (PBS only) mice grew CT26 and CT26hghE2t tumors that remained present throughout the experiment (data not shown). In mice that were vaccinated with VSVΔG or VSVΔG-C/E1/E2, all animals (8/8 per group) that received control CT26 challenge developed tumors by day 8 (Fig. 7A and B). Tumor measurements demonstrated that wild-type CT26 cancer grew exponentially from day 8 to day 20 in all groups without any significant difference between VSVΔG-C/E1/E2- and VSVΔG-immunized mice (Fig. 7A and B). Median CT26 tumor diameters in VSVΔG- and VSVΔG-C/E1/E2-vaccinated mice were similar on day 8 (5.18 versus 5.4 mm, respectively) and increased rapidly over the course of the experiment (13.7 mm for VSVΔG and 13.2 mm for VSVΔG-C/E1/E2 by day 20) (Fig. 7E). These tumors remained present throughout the experiment, and together with the PBS-treated mice, these mice were eventually sacrificed due to tumor size. All VSVΔG-immunized mice also presented CT26-hghE2t tumors between days 8 and 14 postchallenge. However, two animals in this group were found to clear the tumor (Fig. 7C). Significantly, only 3/8 animals inoculated with VSVΔG-C/E1/E2 mice exhibited any form of tumor by day 26 (Fig. 7D). Furthermore, the median CT26-hghE2t tumor diameter of VSVΔG-immunized mice was much greater (12 mm) than that in VSVΔG-C/E1/E2-vaccinated mice (2.31 mm) (Fig. 7F). In addition, mice that had been immunized with VSVΔG and VSVΔG-C/E1/E2 showed a significant difference ($P = 0.00039$) in the tumor growth kinetics over time between these two groups following CT26hghE2t challenge (as determined by statistical analysis using Student's t test). Our data indicate a significant correlation for protection against CT26-hghE2t challenge in mice immunized with VSVΔG-C/E1/E2.

To investigate whether tumor rejection could involve VSVΔG-C/E1/E2-generated CTL activity to E2, which could eliminate CT26-hghE2t growth, IFN-γ ELISPOT analysis was carried out in vaccinated mice. For the CT26-hghE2t analysis, two surviving mice from the VSVΔG-C/E1/E2 or VSVΔG group that had been immunized as described above and challenged with CT26-hghE2t tumor cells were sacrificed on day 30. Splenocytes from mice were stimulated with mitomycin C-treated CT26-hghE2t cells (or the irrelevant HeLa cell control) at a ratio of 10:1 splenocytes to cancer cells for 1 week. For E2-specific CTL analysis, splenocytes were stimulated with E2 peptide as previously described (Fig. 7G). This analysis showed that while VSVΔG-C/E1/E2 mice had slightly elevated levels of CT26-hghE2t IFN-γ-producing splenocytes, larger tumor-bearing VSVΔG-immunized mice also exhibited IFN-γ reactivity to CT26-hghE2t cancer for reasons that remain unclear. However, it is possible that VSVΔG exerts oncolytic activity to lyse tumor cells and generate some CTL activity to E2 (4, 5, 25). Interestingly, there was a clear difference in the level of CTL activity to E2 peptide, which may have accounted for enhanced protection in VSVΔG-C/E1/E2 mice compared with the VSVΔG group (Fig. 7G). Immunoblot analysis of CT26-hghE2t cells was performed and confirmed expression of HCV E2 glycoprotein prior to insertion of the cancer cells into mice (Fig. 7H). These results indicate that CTL-mediated IFN-γ activity to CT26-hghE2t cells can occur in mice challenged with this tumor and, more specifically, that E2 CTL activity generated from VSVΔG-C/E1/E2-immunized mice may facilitate protection against HCV-associated tumor formation.

VSVΔG-C/E1/E2 immunization of BALB/c mice protects against vvHCV.S challenge. The above data indicated that VSVΔG-C/E1/E2 immunization could protect against a tumor model expressing HCV E2. Subsequently we expanded our studies to examine the potential use of our vaccine strategy to neutralize a surrogate viral HCV challenge model. Mice immunized as described above for the tumor experiments were challenged with 1×10^7 PFU of recombinant vaccinia virus-expressing HCV-C/E1/E2 (vvHCV.S) or with a control vaccinia virus–wild-type Western Reserve strain (vv-WR) (39, 51). Intraperitoneal injection of vvHCV.S or vv-WR was undertaken 2 weeks following the last boost with VSVΔG-C/E1/E2 or VSVΔG. Control PBS-injected mice were also administered the same dose of challenge virus. Five days later, ovaries were harvested from mice and freeze-thawed several times subsequent to plaque titer analysis (Fig. 8A). The mean (five mice per group) vvHCV.S titer observed in control PBS mice was 2.28×10^9 PFU/ml, and that for VSVΔG-immunized mice was 2.98×10^9 PFU/ml. However, in mice immunized with VSVΔG-C/E1/E2, the mean vvHCV.S titer was 1.18×10^7 PFU/ml and was 2 logs lower than that in VSVΔG-immunized mice ($P = 0.0039$) and PBS-treated mice ($P = 0.010$). There

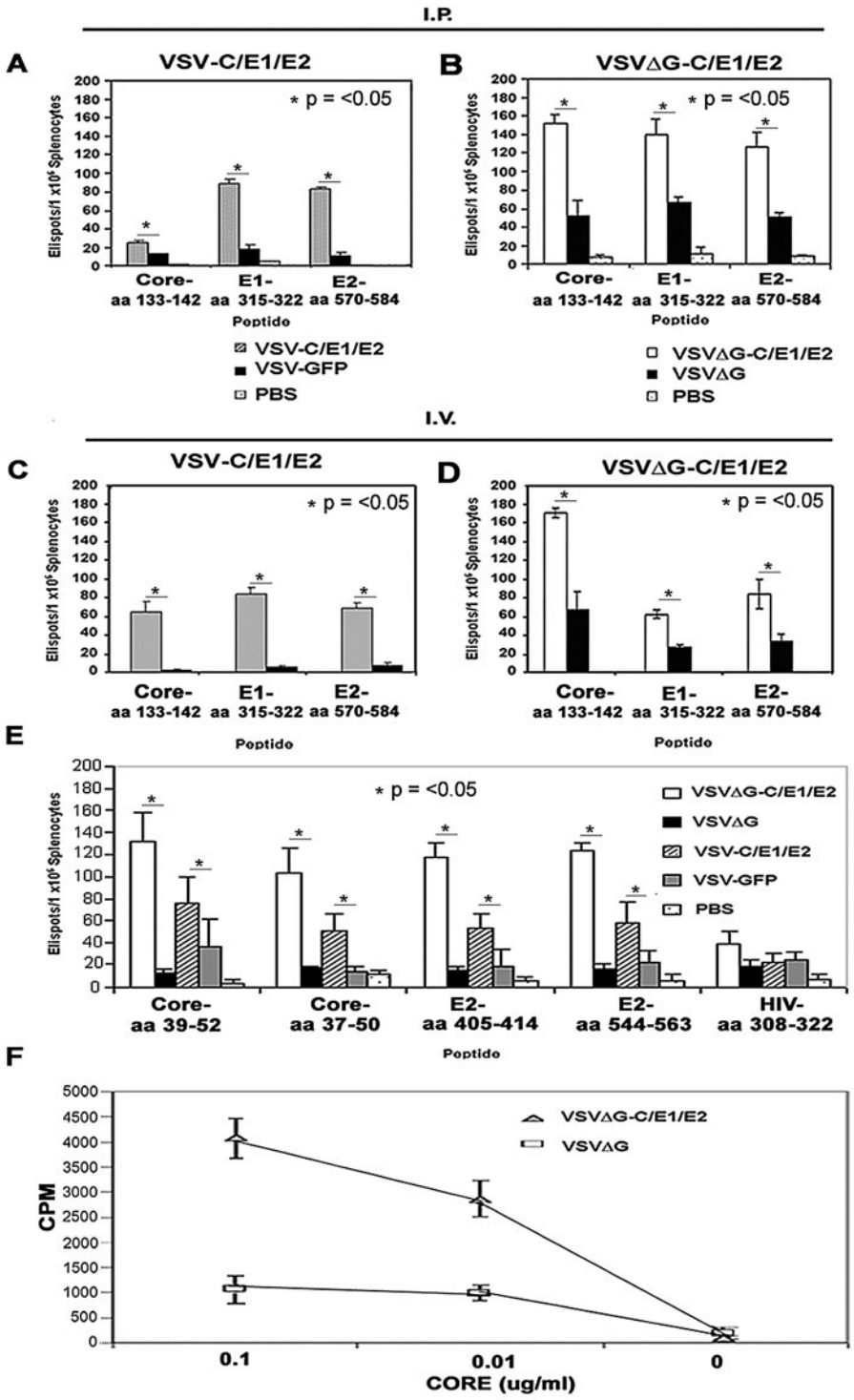

FIG. 6. HCV-specific T-cell responses to core, E1, or E2. Splenectomies were performed on BALB/c mice sacrificed (day 21) following vaccination as described for antibody responses with VSV-C/E1/E2 or VSV-GFP and VSVΔG-C/E1/E2 or VSVΔG by the i.p. route (A and B, respectively) or i.v. injection (C and D). PBS controls were also included. Splenocytes were harvested from three mice per group, pooled, and plated in a precoated anti-mouse IFN-γ MAb 96-well format at dilutions of 1×10^6 to 0.25×10^6 (in triplicate) in RPMI medium containing IL-2 and HCV peptide as shown. Unstimulated (mock) splenocytes were also plated for each group. After 36 h at 37°C, ELISPOT analysis was performed (described in Materials and Methods). The results are presented as mean ELISPOTS per million splenocytes − any background ELISPOTS from unpulsed mock controls ± standard deviation from the means. *P* values represent Student's *t* test analysis of comparisons of the ELISPOTS between virus groups. Splenocytes were isolated from BALB/c mice immunized intraperitoneally (five per group) as described above and tested against a panel of additional CTL epitopes in HCV C or E2 (E). A control HIV peptide was also included. Lymphoproliferative responses in VSVΔG-C/E1/E2- or VSVΔG-immunized mice were also measured (as described in text) against HCV core 1a antigen (F). The results represent cpm as means of triplicate values at each cell concentration (error bars represent standard deviation).

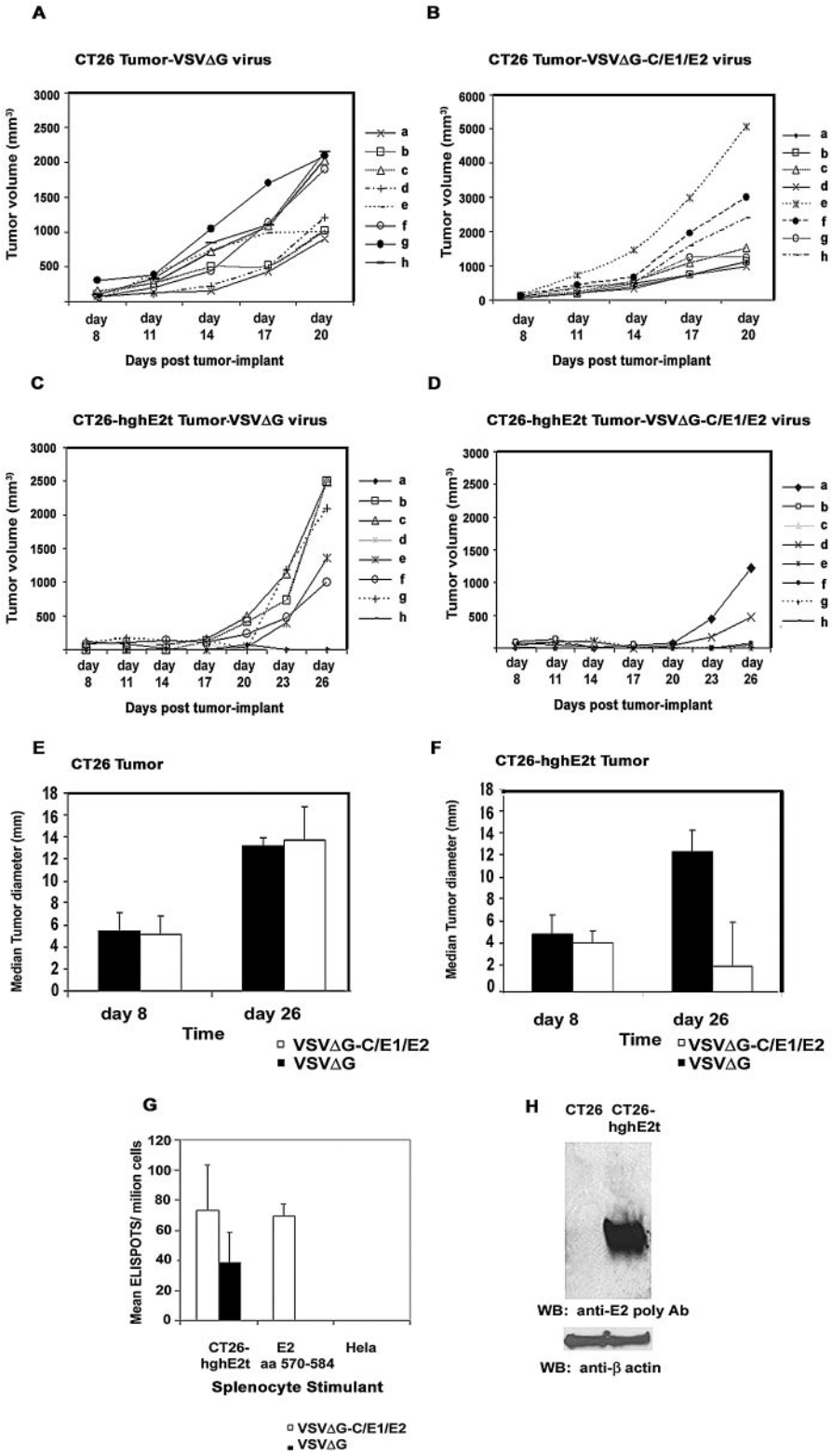

FIG. 7. VSVΔG-C/E1/E2 vaccination protects against HCV E2-expressing CT26-hghE2t tumor. BALB/c mice (eight per group) were vaccinated with three injections 6×10^6 PFU of VSVΔG or VSVΔG-C/E1/E2 intravenously (days 1, 14, and 28). Two weeks following the second boost, mice were challenged with 2×10^6 wild-type CT26 cancer cells injected subcutaneously into mice immunized with VSVΔG or VSVΔG-C/E1/E2 (A and B, respectively). VSVΔG- or VSVΔG-C/E1/E2-immunized mice were also challenged with HCV-E2-expressing (CT26hghE2t) cancer cells (C and D, respectively). The tumors were observed at 3-day intervals, and the results are presented as tumor volumes (in cubic millimeters) for each mouse. The median CT26 and CT26-hghE2t tumor diameters (E and F, respectively) are shown for the first and last measurements for each challenged group. (G) IFN-γ ELISPOT analysis in CT26-hghE2t-challenged mice. Splenectomies were performed on VSVΔG-C/E1/E2 (or

were no differences in viral titers in any of the groups who were challenged with a nonspecific vv-WR (Fig. 8B). These findings indicate that the VSV-expressed HCV structural antigens can inhibit the replication of vvHCV.S in VSVΔG-C/E1/E2-immunized mice. The specificity of this protection is illustrated by the lack of neutralization against vv-WR challenge in VSVΔG-C/E1/E2-immunized mice (Fig. 8B). We also observed vvHCV.S virus titer reduction in animals immunized with VSV-C/E1/E2 (Fig. 8C). The experiments were performed as described above for VSVΔG-C/E1/E2, except that we also evaluated the potential effect of ovary size against final vvHCV.S titers. We found that in VSV-C/E1/E2-immunized animals, there was over a 2-log reduction ($P = 0.022$) in the ratio of residual vvHCV.S titers per milligram of ovaries as compared to animals immunized with VSV-GFP. The results are indicative of the protective immune responses induced by VSV-C/E1/E2 or VSVΔG-C/E1/E2 and are not due to variations in weights of ovaries sampled. Collectively, our data indicate that VSVΔG-C/E1/E2 could present a promising and safer alternative strategy for immunotherapy against HCV.

DISCUSSION

A nonpropagating vesicular stomatitis virus encoding the HCV structural proteins (VSVΔG-C/E1/E2) was generated and evaluated as a safer alternative vaccine strategy in murine models. We report that VSVΔG-C/E1/E2 expressed high levels of the HCV core, E1, and E2 structural proteins, a significant quantity of which were authentically processed as noncovalently linked HCV E1/E2 oligomers, as demonstrated by binding to conformationally sensitive mouse or human MAbs. In this report, we utilized several previously described mouse or human conformation-dependent antibodies to examine the authenticity of VSV-expressed HCV glycoproteins (18, 26, 32, 57). These antibodies included human mAbs CBH-7 and CBH8-C, which block binding of E2 to the cellular receptor CD81 (32). CBH-7 and CBH8-C were isolated from an HCV 1b-infected individual and have been reported as being a positive indicator for the correct processing of VSV-expressed HCV E1/E2 noncovalent heterodimers. Such data would indicate that VSV-expressed HCV glycoproteins may generate humoral responses that could be effective at preventing bona fide HCV infection, at least against similar genotypes. Furthermore, our findings that VSV-induced anti-HCV E2 antibodies were cross-reactive between genotypes 1a and 1b is encouraging for vaccine purposes, since these genotypes are the most prevalent worldwide (45). Interestingly, nonpropagating VSVΔG-C/E1/E2 was found to stimulate humoral and cellular immune responses in mice that were comparable to those of live VSV-C/E1/E2 without the prerequisite for adjuvant enhancement. This strategy has several features that are beneficial over replication-competent approaches and seem to generate robust immune responses. First, the removal of the VSV-G gene from the genome results in the reduction of the highly antigenic VSV glycoprotein, allowing immune responses to respond more efficiently against HCV structural antigens. Second, this technology has the benefit of being a safer viral approach that still can initiate both humoral and cellular immunity. Although nonpropagating, it is likely that this viral system may still facilitate innate intracellular responses that control subsequent adaptive immunity through release of cytokines and "cross-priming." Current, E1/E2 subunit vaccines appear to generate humoral responses but lack the capability of inducing CTL responses (14, 37, 60). However, core protein-based nonclassical immunostimulating complex approaches have been shown to generate long-lived $CD4^+$ and $CD8^+$ Th0-type responses in rhesus macaques (60). Although currently our studies in BALB/c mice are in preliminary stages, the humoral and cellular immune responses against HCV structural proteins using a nonpropagating VSV approach provide an excellent platform for further studies to enhance and review the immune response further in both transgenic human HLA-A 2.1 (AAD) mice and nonhuman primate models.

To date, vaccine approaches to HCV have failed to produce sterilizing immunity, and with this factor in mind, here we utilized two surrogate challenge models to examine the protective nature of the nonpropagating VSVΔG-C/E1/E2 strategy using mouse models. In the first challenge model, we utilized a CT26-hghE2t mouse colon cancer model expressing HCV-E2. VSVΔG-C/E1/E2-immunized mice appeared protected from CT26-hghE2t tumors since the growth of the cancer was restricted compared to that in control groups and CT26 tumors. This effect was clearly due to E2 immunization since control groups were not protected against CT26-hghE2t tumors and there was no protection against wild-type CT26 challenge. Furthermore, ELISPOT analysis illustrated that $CD8^+$ CTL activity specific to E2 may in part be responsible for this protection. However, we cannot rule out antibody-dependent cell-mediated cytotoxicity, a process frequently mediated by IgG2a binding to FcγR on macrophages and natural killer cells (1, 41, 73). In our study, we also observed IgG2a responses to HCV core and anticipate that similar responses to HCV E2 may be present to facilitate antibody-dependent cell-mediated cellular cytotoxicity to CT26-hghE2t tumor. Intriguingly as VSV exhibits oncolytic activity itself (reviewed in reference 7), it may be interesting to see if replication-competent VSV-C/E1/E2 or even VSVΔG-C/E1/E2 can be used therapeutically to treat animals bearing CT26-hghE2t tumors. Whether through immune enhancement or potentially by direct oncolytic activity, these studies may provide a basis for feasibility studies into HCV cancer vaccines against potentially HCV-induced B-cell non-Hodgkin's lymphomas or hepatocellular carcinoma (19, 54, 67).

The only true infectious in vivo model organism with which to examine HCV vaccines before trials in humans is the chim-

VSVΔG)-vaccinated mice (two mice from each group) at day 26 post-tumor challenge. Responder splenocytes were stimulated with target CT26-hghE2t cells or negative control HeLa cells at a ratio of 10:1 (dilutions in triplicate) for 1 week at 37°C prior to ELISPOT analysis as described in Materials and Methods. The results are presented as mean ELISPOTS (subtracted from mock unpulsed controls per group) ± standard deviation from the mean. (H) Western blot (WB) analysis of CT26 and CT26-hghE2t cell lines was performed using anti-E2 polyclonal antibody (poly Ab) prior to injection into mice. The blots were reprobed with anti-β-actin MAb.

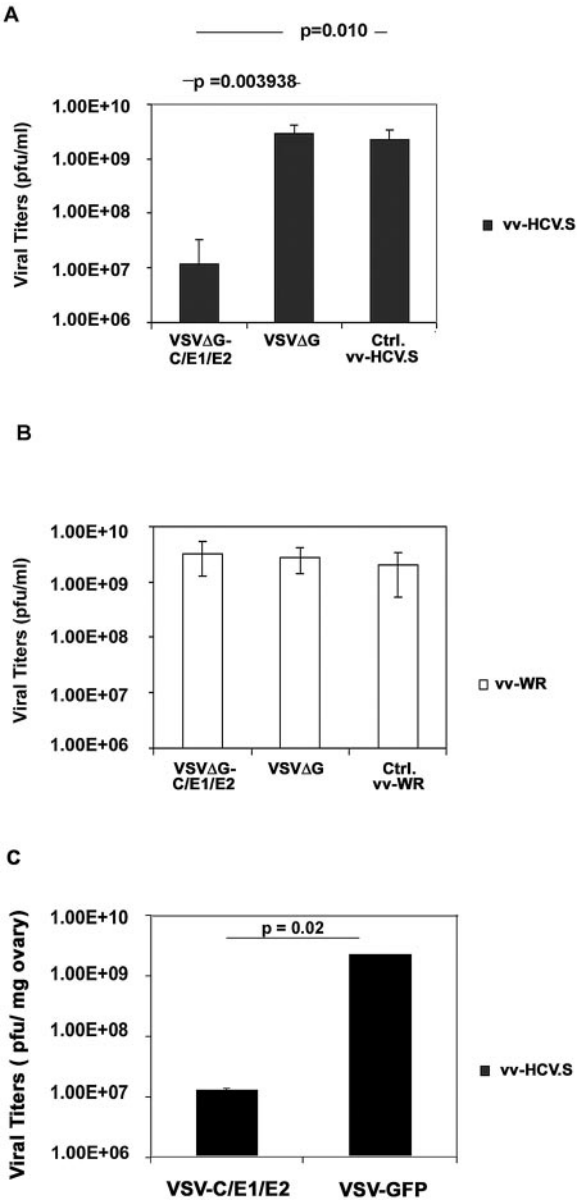

FIG. 8. VSVΔG-C/E1/E2 immunization of BALB/c mice inhibits viral replication of recombinant vaccinia virus (vvHCV.S) expressing HCV structural antigens. (A) Mice (five per group) were vaccinated intravenously (days 1, 14, and 28) with 6×10^6 PFU/ml of VSVΔG or VSVΔG-C/E1/E2 and 2 weeks later challenged with 1×10^7 PFU/ml of vvHCV.S administered by intraperitoneal injection. A nonimmunized mouse group also received the same dose of vvHCV.S. Ovaries were harvested from all mice on day 5 (peak viral titers), and following homogenization and freeze-thaw, viral titers were evaluated by plaque assay. The mean viral titers (error bars represent standard deviations) for each group are given. (B) Viral challenge (1×10^7 PFU/ml) with control vaccinia virus Western Reserve strain (vv-WR). The experiment was performed in parallel and as described above. (C) VSV-C/E1/E2- or VSV-GFP-immunized animals (as above) were also challenged with 1×10^7 PFU/ml of vvHCV.S administered by intraperitoneal injection. The ovaries were harvested on day 5, measured (mg), and processed as described above followed by plaque assay to evaluate viral titers. The results represent mean viral titers expressed as a ratio of residual virus and size of ovary. *P* values represent Student's *t* test analysis of comparisons of viral titers between groups.

panzee. However, the ability to titrate recombinant vvHCV.S or vv-WR in murine ovaries offers another useful measure of protective immunity by potential prophylactic HCV vaccines. In this study, we noticed a significant reduction in vvHCV.S plaque titers of mice that had been immunized with VSVΔG-C/E1/E2 compared to VSVΔG- or PBS-immunized control groups. The lack of complete neutralization of vvHCV.S may be due to the heterologous nature of the recombinant viral challenge. However, others have demonstrated the usefulness of recombinant vaccinia virus challenge models in improving vaccine approaches in mice and nonhuman primates against HCV and human papillomavirus (48, 51). The goals for vaccine approaches are to potentially achieve optimal humoral and cellular (strong $CD4^+$ and $CD8^+$) responses to engage HCV infection before infection can outpace the immune system, as seems to occur during persistence (79). Additional hope comes from the fact that HCV immunotherapy may prevent chronic infection and also liver disease that appears to be associated with potentially compromised T-cell responses and their effector functions (63). Using our experimental models, we describe positive humoral and cellular (total lymphocyte proliferation and CD8) immune responses that include markers associated with Th1-type (IgG2a and IFN-γ) immunity. Since these responses play an important role in resolving HCV infection (15, 30, 75), our findings here and subsequent observations from two different challenge systems provide the basis for further study of VSVΔG-C/E1/E2-based vaccination and immunotherapy to combat HCV infection.

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REFERENCES

1. **Adams, D. O., T. Hall, Z. Steplewski, and H. Koprowski.** 1984. Tumors undergoing rejection induced by monoclonal antibodies of the IgG2a isotype contain increased numbers of macrophages activated for a distinctive form of antibody-dependent cytolysis. Proc. Natl. Acad. Sci. USA **81:**3506–3510.
2. **Afdhal, N. H.** 2004. The natural history of hepatitis C. Semin. Liver Dis. **24**(Suppl. 2)**:**3–8.
3. **Agnello, V., A. Gyorgy, M. Elfahal, G. B. Knight, and Q.-X. Zhang.** 1999. Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96:**12766–12771.
4. **Balachandran, S., and G. N. Barber.** 2000. Vesicular stomatitis virus (VSV) therapy of tumors. IUBMB Life **50:**135–138.
5. **Balachandran, S., M. Porosnicu, and G. N. Barber.** 2001. Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or Myc function and involves induction of apoptosis. J. Virol. **75:**3474–3479.
6. **Ball, L. A., C. R. Pringle, B. Flanagan, V. P. Perepelitsa, and G. W. Wertz.** 1999. Phenotypic consequences of rearranging the P, M, and G genes of vesicular stomatitis virus. J. Virol. **73:**4705–4712.
7. **Barber, G. N.** 2004. Vesicular stomatitis virus as an oncolytic vector. Viral Immunol. **17:**516–527.
8. **Bartenschlager, R., and V. Lohmann.** 2000. Replication of the hepatitis C virus. Bailliere's Best Pract. Res. Clin. Gastroenterol. **14:**241–254.
9. **Barth, H., C. Schafer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278:**41003–41012.

10. **Branch, A. D., D. D. Stump, J. A. Gutierrez, F. Eng, and J. L. Walewski.** 2005. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. Semin. Liver Dis. **25:**105–117.
11. **Brinster, C., M. Chen, D. Boucreux, G. Baccala-Paranhos, P. Liljestrom, F. Lemmonier, and G. Inchauspe.** 2002. Hepatitis C virus non-structural protein 3-specific cellular immune responses following single or combined immunization with DNA or recombinant Semliki Forest virus particles. J. Gen. Virol. **83:**369–381.
12. **Bruna-Romero, O., J. J. Lasarte, G. Wilkinson, K. Grace, B. Clarke, F. Borras-Cuesta, and J. Prieto.** 1997. Induction of cytotoxic T-cell response against hepatitis C virus structural antigens using a defective recombinant adenovirus. Hepatology **25:**470–477.
13. **Buonocore, L., K. J. Blight, C. M. Rice, and J. K. Rose.** 2002. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. J. Virol. **76:**6865–6872.
14. **Choo, Q. L., G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, C. Kuo, et al.** 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc. Natl. Acad. Sci. USA **91:** 1294–1298.
15. **Cooper, S., A. L. Erickson, E. J. Adams, J. Kansopon, A. J. Weiner, D. Y. Chien, M. Houghton, P. Parham, and C. M. Walker.** 1999. Analysis of a successful immune response against hepatitis C virus. Immunity **10:**439–449.
16. **Cormier, E. G., R. J. Durso, F. Tsamis, L. Boussemart, C. Manix, W. C. Olson, J. P. Gardner, and T. Dragic.** 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**14067–14072.
17. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**7270–7274.
18. **Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. S. Hahn, C. M. Rice, and J. Dubuisson.** 1997. Formation of native hepatitis C virus glycoprotein complexes. J. Virol. **71:**697–704.
19. **De Re, V., L. Caggiari, R. Talamini, M. Crovatto, S. De Vita, C. Mazzaro, R. Cannizzaro, R. Dolcetti, and M. Boiocchi.** 2004. Hepatitis C virus-related hepatocellular carcinoma and B-cell lymphoma patients show a different profile of major histocompatibility complex class II alleles. Hum. Immunol. **65:**1397–1404.
20. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147–6160.
21. **Dubuisson, J., and C. M. Rice.** 1996. Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. J. Virol. **70:**778–786.
22. **Esumi, M., T. Rikihisa, S. Nishimura, J. Goto, K. Mizuno, Y. H. Zhou, and T. Shikata.** 1999. Experimental vaccine activities of recombinant E1 and E2 glycoproteins and hypervariable region 1 peptides of hepatitis C virus in chimpanzees. Arch. Virol. **144:**973–980.
23. **Ezelle, H. J., D. Markovic, and G. N. Barber.** 2002. Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. J. Virol. **76:**12325–12334.
24. **Farci, P., A. Shimoda, D. Wong, T. Cabezon, D. De Gioannis, A. Strazzera, Y. Shimizu, M. Shapiro, H. J. Alter, and R. H. Purcell.** 1996. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc. Natl. Acad. Sci. USA **93:**15394–15399.
25. **Fernandez, M., M. Porosnicu, D. Markovic, and G. N. Barber.** 2002. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. J. Virol. **76:**895–904.
26. **Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.
27. **French Study Group for the Standardization of Hepatitis C Virus PCR.** 1994. Improvement of hepatitis C virus RNA polymerase chain reaction through a multicentre quality control study. J. Virol. Methods **49:**79–88.
28. **Friedl, P., A. T. den Boer, and M. Gunzer.** 2005. Tuning immune responses: diversity and adaptation of the immunological synapse. Nat. Rev. Immunol. **5:**532–545.
29. **Gerlach, J., H. Diepolder, M.-C. Jung, N. Gruner, W. Schruat, R. Zachoval, R. Hoffman, C. Schirren, T. Santantonio, and G. Pape.** 1999. Recurrence of HCV after loss of virus specific CD4+ T cell response in acute hepatitis C. Gastroenterology **117:**933–941.
30. **Gremion, C., and A. Cerny.** Hepatitis C virus and the immune system: a concise review. Rev. Med. Virol. 2005. **15:**235–268.
31. **Gruner, N. H., J. T. Gerlach, M. C. Jung, H. M. Diepolder, C. A. Schirren, W. W. Schruat, R. W. Hoffman, R. Zachoval, T. Santantonio, M. Cucchiarini, A. Cerny, and G. R. Pape.** 2000. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. J. Infect. Dis. **181:**1528–1536.
32. **Hadlock, K. G., R. E. Lanford, S. Perkins, J. Rowe, Q. Yang, S. Levy, P. Pileri, S. Abrigani, and S. K. H. Foung.** 2000. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J. Virol. **74:**10407–10416.
33. **Hagan, D. T. O., M. Singh, C. Dong, M. Ugozolli, K. Berger, E. Glazer, M. Selby, M. Wininger, P. Ng, K. Crawford, X. Paliard, S. Coates, and M. Houghton.** 2004. Cationic microparticles are a potent delivery system for a HCV DNA vaccine. Vaccine **23:**672–680.
34. **Haglund, K., J. Forman, H. G. Krausslich, and J. K. Rose.** 2000. Expression of human immunodeficiency virus type 1 Gag protein precursor and envelope proteins from a vesicular stomatitis virus recombinant: high-level production of virus-like particles containing HIV envelope. Virology **268:**112–121.
35. **Hallam, N. F., M. L. Fletcher, S. J. Read, A. M. Majid, J. B. Kurtz, and C. R. Rizza.** 1993. Low risk of sexual transmission of hepatitis C virus. J. Med. Virol. **40:**251–253.
36. **Harris, H. E., M. E. Ramsay, N. Andrews, and K. P. Eldridge.** 2002. Clinical course of hepatitis C virus during the first decade of infection: cohort study. Br. Med. J. **324:**450–453.
37. **Houghton, M., Q. L. Choo, D. Chien, G. Kuo, and A. Weiner.** 1997. Development of an HCV vaccination for the induction of immune responses against hepatitis C virus proteins. Vaccine **15:**853–856.
38. **Hussy, P., H. Langen, J. Mous, and H. Jacobsen.** 1996. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology **224:**93–104.
39. **Jeong, S.-H., M. Qiao, M. Nascimbeni, Z. Hu, B. Rehermann, K. Murthy, and T. J. Liang.** 2004. Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates. J. Virol. **78:**6995–7003.
40. **Kahn, J. S., A. Roberts, C. Weibel, L. Buonocore, and J. K. Rose.** 2001. Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge. J. Virol. **75:**11079–11087.
41. **Kipps, T. J., P. Parham, J. Punt, and L. A. Herzenberg.** 1985. Importance of immunoglobulin isotype in human antibody-dependent, cell mediated cytotoxicity directed by murine monoclonal antibodies. J. Exp. Med. **161:**1–17.
42. **Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose.** 1995. Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA **92:**4477–4481.
43. **Lozach, P. Y., A. Amara, B. Bartosch, J. L. Virelizier, F. Arenzana-Seisdedos, F. L. Cosset, and R. Altmeyer.** 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J. Biol. Chem. **279:**32035–32045.
44. **Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278:**20358–20366.
45. **Majid, A., R. Holmes, U. Desselberger, P. Simmonds, and T. A. McKee.** 1995. Molecular epidemiology of hepatitis C virus infection amongst intravenous drug users in rural communities. J. Med. Virol. **46:**48–51.
46. **Majid, A., P. Jackson, Z. Lawal, G. M. Pearson, H. Parker, G. J. Alexander, J. P. Allain, and J. Petrik.** 1999. Ontogeny of hepatitis C virus (HCV) hypervariable region 1 (HVR1) heterogeneity and HVR1 antibody responses over a 3 year period in a patient infected with HCV type 2b. J. Gen. Virol. **80:**317–325.
47. **Majid, A. M., and D. R. Gretch.** 2002. Current and future hepatitis C virus diagnostic testing: problems and advancements. Microbes Infect. **4:**1227–1236.
48. **Marais, D., J. A. Passmore, J. Maclean, R. Rose, and A. L. Williamson.** 1999. A recombinant human papillomavirus (HPV) type 16 L1-vaccinia virus murine challenge model demonstrates cell-mediated immunity against HPV virus-like particles. J. Gen. Virol. **80:**2471–2475.
49. **McHutchison, J. G., and A. T. Dev.** 2004. Future trends in managing hepatitis C. Gastroenterol. Clin. N. Am. **33:**S51–S61.
50. **Mehta, H. S., A. Cox, D. R. Hoover, H.-X. Wang, Q. Mao, S. Ray, S. A. Strathdee, D. Vlahov, and D. L. Thomas.** 2002. Protection against persistence of hepatitis C. Lancet **359:**1478–1483.
51. **Murata, K., M. Lechmann, M. Qiao, T. Gunji, H. J. Alter, and T. J. Liang.** 2003. Immunization with hepatitis C virus-like particles protects mice from recombinant hepatitis C virus-vaccinia infection. Proc. Natl. Acad. Sci. USA **100:**6753–6758.
52. **Obuchi, M., M. Fernandez, and G. N. Barber.** 2003. Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. J. Virol. **77:**8843–8856.
53. **Op de Beek, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F. L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus glycoproteins. J. Virol. **78:**2994–3002.
54. **Pagano, J. S., M. Blaser, M. A. Buendia, B. Damania, K. Khalili, N. Raab-Traub, and B. Roizman.** 2004. Infectious agents and cancer: criteria for a causal relation. Semin. Cancer Biol. **14:**453–471.
55. **Pancholi, P., M. Perkus, N. Tricoche, Q. Liu, and A. Prince.** 2003. DNA immunization with hepatitis C virus (HCV) polycistronic genes or immunization by HCV DNA priming-recombinant canarypox virus boosting induces

immune responses and protection from recombinant HCV-vaccinia virus infection in HLA-A2.1-transgenic mice. J. Virol. **77:**382–390.
56. **Park, S. H., S. H. Yang, C. G. Lee, J. W. Youn, J. Chang, and Y. C. Sung.** 2003. Efficient induction of T helper 1 CD4+ T-cell responses to hepatitis C virus core and E2 by a DNA prime-adenovirus boost. Vaccine **21:**4555–4564.
57. **Patel, A. H., J. Wood, F. Penin, J. Dubuisson, and J. McKeating.** 2000. Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. J. Gen. Virol. **81:**2873–2883.
58. **Patel, J., A. H. Patel, and J. McLauchlan.** 1999. Covalent interactions are not required to permit or stabilize the non-covalent association of hepatitis C virus glycoproteins E1 and E2. J. Gen. Virol. **80:**1681–1690.
59. **Pöhlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070–4080.
60. **Polakos, N. K., D. Drane, J. Cox, P. Ng, M. Selby, D. Chien, D. T. O'Hagan, M. Houghton, and X. Paliard.** 2001. Characterization of hepatitis C virus core-specific immune responses primed in rhesus macaques by a nonclassical ISCOM vaccine. J. Immunol. **166:**3589–3598.
61. **Puig, M., M. E. Major, K. Mihalik, and S. M. Feinstone.** 2004. Immunization of chimpanzees with an envelope protein-based vaccine enhances specific humoral and cellular immune responses that delay hepatitis C virus infection. Vaccine **22:**991–1000.
62. **Ralston, R., K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q.-L. Choo.** 1993. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J. Virol. **67:**6753–6761.
63. **Rehermann, B., and M. Nascimbeni.** 2005. Immunology of hepatitis B virus and hepatitis C virus infection. Nat. Rev. Immunol. **5:**215–229.
64. **Roberts, A., L. Buonocore, R. Price, J. Forman, and J. K. Rose.** 1999. Attenuated vesicular stomatitis viruses as vaccine vectors. J. Virol. **73:**3723–3732.
65. **Roberts, A., E. Kretzschmar, A. S. Perkins, J. Forman, R. Price, L. Buonocore, Y. Kawaoka, and J. K. Rose.** 1998. Vaccination with a recombinant vesicular stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge. J. Virol. **72:**4704–4711.
66. **Roberts, A., J. D. Reuter, J. H. Wilson, S. Baldwin, and J. K. Rose.** 2004. Complete protection from papillomavirus challenge after a single vaccination with a vesicular stomatitis virus vector expressing high levels of L1 protein. J. Virol. **78:**3196–3199.
67. **Roboz, G. J.** 1998. Hepatitis C and B-cell lymphoma. AIDS Patient Care STDs **12:**605–609.
68. **Rose, N. F., P. A. Marx, A. Luckay, D. F. Nixon, W. J. Moretto, S. M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, and J. K. Rose.** 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell **106:**539–549.
69. **Rosen, H. R., C. Miner, A. W. Sasaki, D. M. Lewinsohn, A. J. Conrad, A. Bakke, et al.** 2002. Frequencies of HCV-specific effector CD4+ T cells by flow cytometry: correlation with clinical disease stages. Hepatology **35:**190–198.
70. **Sarih, M., N. Bouchrit, and A. Benslimane.** 2000. Different cytokine profiles of peripheral blood mononuclear cells from patients with persistent and self-limited hepatitis C virus infection. Immunol. Lett. **74:**117–120.
71. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017–5025.
72. **Snapper, C. M., and F. D. Finkelman.** 1999. Immunoglobulin class switching, p. 831–861. *In* W. E. Paul (ed.), Fundamental immunology. Lippincott-Raven Publishers, Philadelphia, Pa.
73. **Song, M. K., S. W. Lee, Y. S. Suh, K. J. Lee, and Y. C. Sung.** 2000. Enhancement of immunoglobulin G2a and cytotoxic T-lymphocyte responses by a booster immunization with recombinant hepatitis C virus E2 protein in E2 DNA-primed mice. J. Virol. **74:**2920–2925.
74. **Tan, S. L., Y. He, Y. Huang, and M. J. Gale, Jr.** 2004. Strategies for hepatitis C therapeutic intervention: now and next. Curr. Opin. Pharmacol. **4:**465–470.
75. **Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari.** 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J. Exp. Med. **194:**1395–1406.
76. **Thursz, M., R. Yallop, R. Goldin, C. Trepo, and H. C. Thomas.** 1999. Influence of MHC class II genotype on outcome of infection with hepatitis C virus. Lancet **354:**2119–2124.
77. **Tsai, S. L., Y. F. Liaw, M. H. Chen, C. Y. Huang, and G. C. Kuo.** 1997. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology **25:**449–458.
78. **Wedemeyer, H., X. S. He, M. Nascimbeni, A. R. Davis, H. B. Greenberg, J. H. Hoofnagle, T. J. Liang, H. Alter, and B. Rehermann.** 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J. Immunol. **169:**3447–3458.
79. **Willberg, C., E. Barnes, and P. Klenerman.** 2003. HCV immunology—death and the maiden T cell. Cell Death Differ. **10**(Suppl. 1)**:**S39–S47.
80. **Yasui, K., T. Wakita, K. Tsukiyama-Kohara, S.-I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J. R. Wands, and M. Kohara.** 1998. The native form and maturation process of hepatitis C virus core protein. J. Virol. **72:**6048–6055.