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Contribution of Virus-Like Particles to the Immunogenicity of Human Immunodeficiency Virus Type 1 Gag-Derived Vaccines in Mice

S. B. Justin Wong[1] and Robert F. Siliciano[1,2]*

Program in Cellular and Molecular Medicine,[1] and Graduate Program in Immunology,[2] Department of Medicine and Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland

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The human immunodeficiency virus type 1 (HIV-1) Gag protein is a major target antigen for cytotoxic-T-lymphocyte-based vaccine strategies because of its high level of conservation. The murine model has been used extensively to evaluate potential HIV-1 vaccines. However, the biology of HIV-1 Gag is somewhat different in human and murine tissues. The ability of HIV-1 Gag to form virus-like particles (VLPs) in human cells is severely curtailed in murine cells. Hence, it is not known whether immunizing mice with expression vectors encoding HIV-1 Gag provides an accurate assessment of the immunogenicity of these candidate vaccines in primates. In this report, we made use of a chimeric Moloney murine leukemia virus (MMLV)–HIV-1 Gag in which the p17 matrix domain of HIV-1 was replaced with the p15 matrix and p12 domains from MMLV. Murine cells expressing this construct released significant amounts of VLPs. The construct preserved *H-2^d*-restricted antigenic determinants in the remaining portion of HIV-1 Gag, allowing immunogenicity studies to be performed with mice. We demonstrated that immunizing mice with plasmid DNA or adenoviral vectors encoding this chimeric Gag did not significantly increase the HIV-1 Gag-specific cellular or humoral immune response when compared to immunization with a myristoylation-incompetent version of the construct. Thus, the release of VLPs formed in vivo may not play a major role in the immunogenicity of vectors expressing HIV-1 Gag constructs.

An effective vaccine against human immunodeficiency virus type 1 (HIV-1) may be the only means of stemming the AIDS epidemic that has developed in many parts of the world. HIV-1 Gag is an attractive target antigen for cytotoxic-T-lymphocyte (CTL)-based vaccine strategies because the sequence is relatively conserved. This sequence conservation has been associated with reports of cross-clade immunity to Gag (17, 39, 61). Gag-specific $CD4^+$- and $CD8^+$-T-cell responses have both been correlated with improved control of viral replication (19, 26, 49), and an ideal HIV-1 vaccine should stimulate both arms of the cellular immune response.

An interesting aspect of Gag biology is that the expression of HIV-1 Gag in certain cells, in the absence of other viral gene products, leads to the spontaneous formation and release of virus-like particles (VLPs) (20). VLPs resemble viral capsids morphologically and antigenically, and VLPs derived from a broad range of viruses have been shown to be immunogenic (5, 10, 14, 15, 21, 28, 29, 32, 35, 41, 45, 50, 52, 59, 60). VLPs are appealing as vaccine candidates because antigen is delivered in a noninfectious form in the absence of viral genome or other potentially toxic viral gene products. Some VLPs are released into the extracellular medium in the form of naked proteinaceous particles, e.g., papillomavirus VLPs. These VLPs may be intrinsically immunogenic because they present repetitive protein arrays which may activate pattern recognition receptors on antigen-presenting cells (33). Other VLPs, like those formed by HIV-1 Gag, are composed of a protein core surrounded by a lipid membrane derived from the host cell during the budding process. This lipid envelope may contain host-derived proteins (18, 43, 57). Immunogenicity studies involving HIV-1 Gag VLPs have been carried out with particles derived from insect cells infected with recombinant baculovirus vectors (45, 59). These studies demonstrated that HIV-1 Gag-specific immune responses can be generated by parenteral administration of Gag VLPs, even without the coadministration of adjuvants. However, the reported immunogenicity of these Gag particles may have been influenced by the presence of foreign proteins on the lipid envelope. It is not clear whether Gag VLPs formed in vivo following immunization with Gag expression vectors would be similarly antigenic, since these would contain Gag cores surrounded by an envelope composed of self lipids and proteins.

Animal models have been used extensively to evaluate the efficacy of candidate HIV-1 vaccines. Rodent models, in particular, have been a popular small-animal model despite the inability of HIV-1 to replicate in rodent tissues. The differences in HIV-1 biology in primate versus rodent tissues is underscored by the numerous barriers that stand in the way of establishing a rodent model of productive HIV infection (2, 3, 9, 31). In particular, when HIV-1 Gag is expressed in murine cells, a defect in plasma membrane targeting leads to the inefficient release of VLPs (36). On the other hand, murine retroviruses like the Moloney murine leukemia virus (MMLV) replicate in murine cells, and MMLV Gag is able to form VLPs when expressed in murine cells (34, 53).

To assess the immunogenicity of HIV-1 Gag-derived VLPs formed in murine tissues in vivo, we have made use of a

* Corresponding author. Mailing address: Department of Medicine, Johns Hopkins University School of Medicine, Broadway Research Building Suite 879, 733 North Broadway, Baltimore, MD 21205. Phone: (410) 955-2958. Fax: (410) 955-0964. E-mail: rsilicia@jhmi.edu.

previously described chimeric MMLV–HIV-1 Gag (13, 47). This construct is able to correctly localize at the plasma membrane, resulting in the assembly and release of HIV-1 Gag-derived VLPs from murine cells. In addition, we created a mutant form of this protein that does not form VLPs in murine cells. By comparing the immune responses generated in mice immunized with these constructs, we were able to investigate the extent to which the in vivo formation of VLPs affects the immunogenicity of HIV-1 Gag. Potentially, this may affect the interpretation of studies of HIV-1 Gag-based recombinant vaccines in rodents when attempts are made to translate these results into estimates of vaccine efficacy in humans.

METHODS AND MATERIALS

Plasmid DNA constructs. MMLV–HIV-1 Gag (MHGag) was created by replacing the p17 matrix domain of HIV-1 Gag with the homologous p15 matrix domain from MMLV (13). In addition, the p12 domain from MMLV Gag, which has no homolog in HIV-1 Gag, was included in the construct. The MMLV p15-p12 segment was amplified with the sense PCR primer 5′-ATTATAGGTA CCATGGGCCAGACTGTTACCACTC-3′, antisense primer 5′-GACTCCACT ACCTCGCAGGCATTCCCCATCGTGCAGAACATCCAGG-3′, and plasmid pMMLVgag-pol carrying the MMLV *gag-pol* gene as a template (a kind gift from Maribeth Eiden, National Institute of Mental Health). Myristoylation of the glycine residue at the amino termini of both MMLV p15 and HIV-1 p17 proteins is essential for membrane association by Gag and virion budding. Deletion of this residue eliminates the ability of Gag to form VLPs (12, 25, 44, 48). To create a version of MHGag with this glycine deleted (abbreviated ΔG-MHGag), the sense primer 5′-ATTATAGGTACCATGCAGACTGTTACCACTCCCTTAAGTTT G-3′ was used instead for PCR, which was otherwise performed as described above. A segment of HIV-1 Gag downstream of matrix was amplified with the sense primer 5′-CCCATCGTGCAGAACATCCAGG-3′, the antisense primer 5′-GCCTGAACAAGATCGTGCG-3′, and plasmid pVRC3900 carrying codon-optimized HXB2 HIV-1 *gag* as a template (a kind gift from Gary Nabel, Vaccine Research Center, National Institutes of Health). The MMLV matrix p12 and HIV-1 Gag PCR products contained complementary regions corresponding to the 5′ end of HIV-1 p24 capsid. These two PCR products were annealed and extended off each other and then digested with Acc65I and SbfI. The plasmid pCiCagPRE was created by replacing non-codon-optimized *gag* in pCigagPRE (62) with *gag* from pVRC3900. pCiCagPRE was digested with Acc65I and SbfI to create the vector into which the MHGag construct was ligated, creating pCiMHGag. In a similar manner, pCiΔG-MHGag was made. In summary, MHGag comprises an amino-terminal component derived from MMLV Gag starting with the amino acid sequence MGQTVTTPLS from p15 and ending with the sequence VADSTTSQAF from p12. This is fused in frame to a carboxy-terminal component derived from codon-optimized HXB2 HIV-1 Gag starting with the amino acid sequence PIVQNIQGQM from p24 capsid and ending with the sequence SLFGSDPSSQ from p6 (Fig. 1A), preserving the *H-2K*^{d}-restricted epitope, AMQMLKETI, found in HIV-1 p24.

Adenovirus constructs. Recombinant E1/E3-deleted adenoviral vectors expressing MHGag (Ad5-MHGag) or ΔG-MHGag (Ad5-ΔG-MHGag) were created using the Adeno-X expression system 2 from BD Clontech. Briefly, MHGag or ΔG-MHGag was cloned into the donor plasmid, pDNR-CMV. The insert was subsequently introduced into the adenoviral acceptor vector pLP-Adeno-X-CMV by Cre-LoxP-mediated site-specific recombination and amplified in *Escherichia coli*. Recombinant adenoviral vector plasmids were screened for the presence of insert and absence of nonrecombinants and transfected into 911 cells to generate a crude viral stock. This viral stock was subsequently amplified in 911 cells and purified by double banding on cesium chloride, and titers were determined by plaque-forming assay with 911 cells.

Cell culture. C2C12 and 911 cells were maintained in Dulbecco's minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin, and streptomycin. In addition, medium for the growth of C2C12 cells was supplemented with 1 mM sodium pyruvate (Invitrogen). P815 and Pvge cells (62) were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, penicillin, and streptomycin.

Western blots. C2C12 cells were transfected with plasmid DNA by using Lipofectamine 2000 reagent (Invitrogen) according to the suggested protocol. Seventy-two hours after transfection, the supernatant was collected, clarified by centrifuging at 300 × *g* for 10 min, passed through a 0.22-μm-pore-size polyvinylidene difluoride (PVDF) filter, and ultracentrifuged at 25,000 × *g* (Contifuge 17 RS; Heraeus) through a 20% sucrose cushion (20% [wt/vol] sucrose in 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100 mM NaCl) for 90 min at 4°C to pellet the particulate matter. This pelleted material derived from the culture supernatant and cell samples were lysed in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen), run under reducing conditions through a 4 to 12% Bis-Tris gradient gel (Invitrogen), transferred onto a PVDF membrane (Immobilon; Millipore), blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20, immunoblotted with polyclonal anti-HIV-1 human serum, and developed with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) secondary antibody (Bio-Rad) and chemiluminescence reagents (Perkin Elmer Life Sciences).

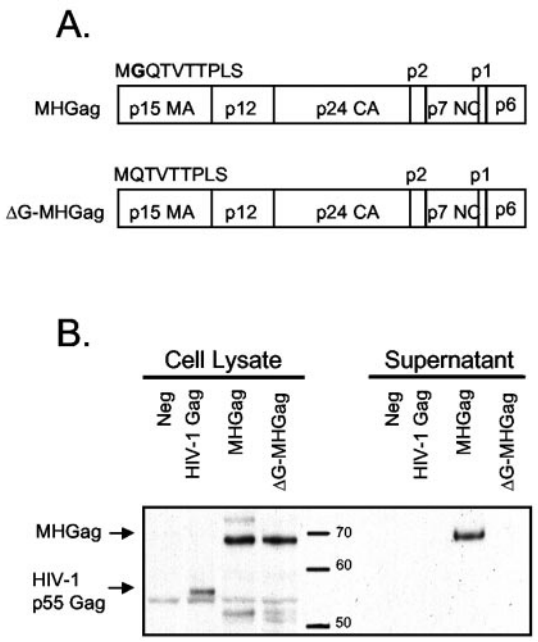


FIG. 1. Map of MMLV–HIV-1 chimeric Gag constructs and their expression in C2C12 cells. (A) Schematic representation of MHGag and ΔG-MHGag. The single-letter designations for the amino-terminal residues of both constructs have been depicted. The p15 and p12 domains from MMLV *gag* were fused in frame with the codon-optimized domains from HIV-1 *gag* encoding p24, p2, p7, p1, and p6. The ΔG-MHGag ORF was made in a similar fashion, but the glycine residue at position 2 of the p15 domain was deleted. Both were inserted into the pCI plasmid backbone for in vitro expression and in vivo immunization studies. (B) Western blot analysis. C2C12 murine myoblasts were transfected with the plasmids pCiEGFP (negative control), pCiCagPRE (codon-optimized HIV-1 Gag), pCiMHGag, or pCiΔG-MHGag. Protein expression in the cell lysate and ultracentrifuged culture supernatant were analyzed by Western blotting with polyclonal human anti-HIV-1 serum. The molecular mass of MHGag was calculated to be approximately 65 kDa. Neg, negative.

Mice. Five- to six-week-old female BALB/c mice were obtained from either the National Cancer Institute or Harlan Sprague-Dawley and housed under specific-pathogen-free environments. All animal work was performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins School of Medicine.

Immunization with plasmid DNA expression vectors. In DNA prime-vaccinia virus boost experiments, the mice were primed by intramuscular immunization with 100 μg of plasmid DNA prepared by Endofree plasmid mega kits (QIAGEN). Three to four weeks later, these mice were challenged intravenously with 3×10^6 PFU of vGag, a recombinant vaccinia virus expressing HIV-1 Gag (62). Three days after this challenge, the mice were sacrificed and assays of lymphocyte function were carried out as described below. In other experiments, mice were immunized by intramuscular injection with 100 μg of plasmid DNA three times at 2-week intervals. Three to four weeks after the last immunization, the mice were sacrificed and assays of immune function were performed.

Chromium release assay. Spleens from immunized mice were harvested and homogenized by passage through a 70-μm-pore-size mesh filter (BD Falcon). The resulting splenocyte suspension was washed and resuspended in ACK red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA [pH 7.2]; Biosource International, Rockville, Md.), then washed twice, and resuspended in RPMI 1640 (Invitrogen) supplemented with penicillin-streptomycin (Invitrogen), 10% fetal calf serum, and 2-mercaptoethanol (Invitrogen). P185 and Pvge target cells were labeled with 100 μCi of ^{51}Cr (Amersham) at 37°C for 2 h. The latter cells stably express HIV-1 Gag-green fluorescent protein fusion protein. Radiolabeled targets were washed three times, diluted to 2.5×10^4 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, 2-mercaptoethanol, penicillin, and streptomycin, and plated at 100 μl per well in 96-well V-bottom plates. Freshly harvested effector splenocytes from vaccinated mice were added to quadruplicate wells at the indicated effector-to-target-cell ratios and incubated for 4 to 5 h at 37°C in a humidified incubator with 5% CO_2. Fifty microliters of supernatant was removed from each well and assayed on a Lumaplate with a TopCount solid-state scintillation counter (Packard). Percent specific lysis was calculated by using the formula $100 \times$ [(experimental lysis − media lysis)/(NP-40 lysis − media lysis)]. The figures show net specific lysis, where the background lysis of P815 cells has been subtracted from the lysis of Pvge targets.

In vivo CTL assay. Splenocytes (5×10^8) from naive syngeneic BALB/c mice were labeled for two rounds with PKH26 dye (Sigma) according to the manufacturer's instructions, except for the following changes. During the first round of labeling, the cells were resuspended in 2.5 ml of diluent C and mixed with 4 μM PKH26 dye diluted in 2.5 ml of diluent C so that the cells were labeled at a final dye concentration of 2 μM. During the second round of labeling, a final concentration of 10 μM PKH26 was used to label the cells. The PKH26-labeled cells were divided into two groups. One fraction was pulsed with the *H-2K*^{d}-restricted HIV-1 Gag peptide P7G (AMQMLKETI) for 2 h at 37°C in a humidified incubator containing 5% CO_2. This fraction was subsequently labeled with 5 μM 5 (and 6)-carboxyfluorescein diacetate (CFSE) (Molecular Probes). The other fraction was left unpulsed and labeled with 0.5 μM CFSE. The efficacy of dye labeling was assessed by flow cytometry before the target cells were injected into mice. A total of 10^7 cells in 100 μl of Hanks balanced salt solution (Invitrogen), comprising equal numbers of peptide-pulsed and unpulsed cells, were injected intravenously into the tail vein of each mouse. The spleens of recipient mice were harvested 15 h later, and single-cell suspensions of splenocytes were assessed by flow cytometry. The infused target cells were distinguished from recipient splenocytes by gating on PKH26 dye-positive cells. High-CFSE peptide-pulsed target cells and low-CFSE control cells were each expressed as a percentage of the total number of PKH26-positive cells. The percentage of specific killing was obtained by using the following equation: 100 − ([percentage of high-CFSE PKH26$^+$ cells/percentage of low-CFSE PKH26$^+$ cells] × 100).

Lymphocyte proliferation assay. Effector splenocytes were resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2-mercaptoethanol. Cells (2×10^5) were seeded in quadruplicate into round-bottom 96-well microtiter plates and stimulated with 2.5 μg of recombinant HIV-1 p24 (Protein Sciences Corp.)/ml or control baculovirus protein. Cells were also stimulated with 1 μg of anti-mouse CD3e antibody (clone 500A2; BD Pharmingen)/ml as a positive control. The cells were incubated at 37°C in 5% CO_2. On the third day, 1 μCi of tritiated thymidine was added to each well, and the cells were incubated for a further 12 h before being harvested. The incorporation of tritiated thymidine was measured with a β-particle plate reader, and the results were expressed as both stimulation indices and net counts per minute.

Flow cytometry. For intracellular staining of IFN-γ production, 5 million effector splenocytes in 200 μl of culture medium (RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2-mercaptoethanol) were plated in each 96-well plate and stimulated for 4 to 5 h with or without 10 μM P7G peptide in the presence of Golgistop (BD Pharmingen). Alternatively, the splenocytes were incubated overnight with 5 μg of recombinant HIV-1 Gag p24 capsid protein (Protein Sciences Corp)/ml or 5 μg of control baculovirus protein/ml, after which Golgistop was added for a further 4 h. The splenocytes were then fixed and permeabilized with Cytofix/Cytoperm reagent (BD Pharmingen) according to the manufacturer's instructions. Permeabilized splenocytes were stained with fluorescein isothiocyanate-conjugated rat anti-mouse gamma interferon (IFN-γ) (clone XMG1.2; BD Pharmingen) and phycoerythrin-conjugated rat anti-mouse CD8a (clone 53-6.7; BD Pharmingen) or phycoerythrin-conjugated rat anti-mouse CD4 (clone GK1.5; BD Pharmingen). In some experiments, CD8 cells were additionally stained with peridinin chlorophyll protein-conjugated Armenian hamster anti-mouse CD3e (clone 145-2C11; BD Pharmingen). The stained cells were analyzed with either a FACScan or FACSCalibur flow cytometer and CellQuest Software (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA) for anti-Gag antibodies. Blood was collected from the sacrificed animals and processed with Microtainer serum separator tubes (Becton Dickinson). Costar 96-well flat-bottom enzyme immunoassay plates (Corning) were precoated with 50 μl of 1-μg/ml HIV-1 Pr55 Gag solution (Protein Sciences) in PBS and stored overnight at 4°C. The plates were rinsed with water, and residual binding capacity was blocked with buffer containing 0.015 M sodium borate, 0.12 M sodium chloride, 1 mM EDTA, 0.05% (vol/vol) Tween 20, and 0.25% (wt/vol) bovine serum albumin. After rinsing off the blocking buffer, 50 μl of serum samples serially diluted 10-fold in blocking buffer was added to the wells. A standard curve was also constructed by using serial dilutions of monoclonal mouse anti-HIV-1 p24 antibody (Dako). After overnight incubation at 4°C, the coated plates were rinsed with water and blocked with buffer again. Horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chains) (Bio-Rad) was diluted 1:10,000 in blocking buffer, and 50 μl of this solution was added to each well. The plate was left to stand for 2 h at room temperature before being rinsed with water and blocked with buffer. Fifty microliters of 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma) was added to each well for 30 min before the reaction was stopped with 50 μl of 4 N sulfuric acid. Substrate hydrolysis was quantified with a microtiter plate reader.

Generation of bone marrow-derived dendritic cells. Bone marrow dendritic cells were generated as previously described (27). Briefly, marrow from the femora and tibiae of 5- to 6-week-old BALB/c mice was isolated, and the red blood cells were lysed with ACK lysis buffer (Biosource International). The resultant cells were seeded in six-well plates at a density of 6 million cells per well and cultured for 6 days in the presence of DC medium (RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES buffer, 50 μM 2-mercaptoethanol, 4 ng of interleukin-4 [Peprotech]/ml, 9 ng of granulocyte-macrophage colony-stimulating factor [Peprotech]/ml, penicillin, and streptomycin) with gentle rinsing and medium replacement every 2 days to dislodge nonadherent cells. On the sixth day of culture, free floating cells were recovered from the culture and CD11c$^+$ cells were purified by using magnetically labeled microbeads according to the manufacturer's instructions (Miltenyi).

Adenoviral infection of bone marrow-derived dendritic cells. Purified CD11c$^+$ bone marrow-derived dendritic cells were seeded at a density of 10^5 cells/well in 48-well plates. These cells were infected at a multiplicity of infection (MOI) of 250 with the E1- and E3-deleted adenoviral vectors vAX β-galactosidase (BD Clontech), Ad5-MHGag, or Ad5-ΔG-MHGag. Dendritic cells were assayed for the presence of intracellular HIV-1 p24 by flow cytometry 2 days later. The cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) and stained with 5 μl of a 1:250 dilution of RD1-conjugated anti-HIV-1 p24 antibody (clone KC57; Beckman Coulter) together with 5 μl of a 1:10 dilution of allophycocyanin-conjugated anti-CD11c antibody (clone HL3; BD Pharmingen). Four days after infection, culture supernatant from infected dendritic cells was clarified by centrifugation at $300 \times g$ for 10 min, filtered through a 0.22-μm-pore-size PVDF filter, and ultracentrifuged at $25{,}000 \times g$ for 90 min through a 20% sucrose cushion to concentrate particulate material. This material was analyzed by immunoblotting as described above.

EM. Transmission electron microscopy (EM) imaging was performed at the Johns Hopkins University School of Medicine Microscope Facility. CD11c$^+$ bone marrow-derived dendritic cells were infected with Ad5-MHGag at an MOI of 500 for 2 days, pelleted, and fixed with 2% glutaraldehyde in buffer containing 0.1 M cacodylate and 3 mM $CaCl_2$. The sample was postfixed with 1% reduced osmium tetroxide in 0.2 M cacodylate buffer containing potassium ferrocyanide and stained with 2% uranyl acetate. The stained sample was dehydrated in an ethanol series and infiltrated with Spurrs resin prior to sectioning.

Immunization with transduced dendritic cells. CD11c$^+$ bone marrow-derived dendritic cells were infected with vAX-enhanced green fluorescent protein (EGFP) (BD Clontech), Ad5-MHGag, or Ad5-ΔG-MHGag at an MOI of 250. Two days later, these cells were thoroughly washed and resuspended in Hanks' balanced salt solution (Invitrogen). BALB/c mice were subsequently immunized with these cells. Approximately 10^4 cells were intravenously injected into each mouse. These mice were allowed to rest for 3 to 4 weeks before receiving a boost of 3×10^6 PFU of recombinant vaccinia virus vGag, expressing HIV-1 Gag, delivered by intraperitoneal injection. Three days later, these mice were sacrificed, and the CD8$^+$-T-cell production of IFN-γ in response to stimulation with P7G peptide was assessed by flow cytometry as described above.

Immunization with E1- and E3-deleted adenoviral expression vectors. Mice were immunized intravenously with 5×10^7 PFU of Ad5-MHGag, Ad5-ΔG-MHGag, or adenovirus encoding irrelevant antigen. Three days later, one animal from each group was sacrificed. The livers from these animals were resected, placed in ice-cold PBS containing protease inhibitors, and homogenized by passage across a 70-μm-pore-size cell strainer (BD Falcon). The cell suspension was subsequently disrupted by freeze-thaw and sonication. Samples were lysed in

lithium dodecyl sulfate sample buffer (Invitrogen) and analyzed by Western blotting for HIV-1 Gag as described above. The remaining animals in each group were sacrificed 2 to 3 weeks after immunization to assess the response of splenocytes to stimulation with P7G Gag peptide or HIV-1 p24 by intracellular staining for IFN-γ production as described above.

RESULTS

MMLV–HIV-1 Gag releases particulate antigen when expressed in murine cell lines in vitro. It has been previously demonstrated that murine cells expressing HIV-1 proviral clones in which the p17 matrix domain from HIV-1 Gag was replaced with the p15 matrix and p12 domains from MMLV were able to support plasma membrane localization of Gag in murine cells and release significant amounts of viral particles (13, 47). To explore the immunogenicity of such Gag VLPs in vivo, we generated a high-expression form of this construct by fusing p15 and p12 from MMLV Gag in frame with codon-optimized HIV-1 Gag p24, p2, p7, p1, and p6, creating MHGag (Fig. 1A). Since myristoylation of the glycine residue at position 2 of p15 is essential for particle formation by MMLV (25, 48), a control construct with this glycine deleted, ΔG-MHGag, was also made. Both were inserted into plasmid DNA expression vectors, creating pCiMHGag and pCiΔG-MHGag, respectively. To demonstrate that the expression of MHGag alone in a murine cell line is sufficient for the release of particulate Gag into the supernatant, we performed Western blot analysis of cell lysates and culture supernatant from cells transfected with these constructs. C2C12 myoblasts were transfected with the plasmid pCiCagPRE, expressing codon-optimized HIV-1 Gag, pCiMHGag, pCiΔG-MHGag, or pCiEGFP as a negative control (Fig. 1B). After 72 h, the culture supernatant was clarified by low-speed centrifugation and passage through a 0.22-μm-pore-size filter. This filtrate was subsequently ultracentrifuged through a 20% sucrose cushion to concentrate particulate matter. Although HIV-1 Gag, MHGag, and ΔG-MHGag could be readily immunoblotted in C2C12 cell lysates by using pooled human anti-HIV-1 serum, only supernatant from cells transfected with MHGag contained detectable amounts of particulate antigen that was reactive with the antibody. Note that the additional p12 domain caused MHGag to migrate with a molecular mass of approximately 65 kDa and only full-length unprocessed Gag is detected.

The composition of MHGag preserved the *H-2K*^{d}-restricted HIV-1 Gag epitope, P7G (AMQMLKETI), located in p24 capsid. *H-2*^{d} class II-restricted epitopes in p24 were also preserved. This allowed the immunogenicity of MHGag expression vectors to be investigated in mice.

Antigen-specific immune responses in mice immunized with a DNA prime-vaccinia virus boost regimen. Immunization with vectors encoding either MHGag or ΔG-MHGag would result in the expression of cell-associated antigen. However, only MHGag generates VLPs for release into the extracellular space. To determine whether the formation and release of particulate Gag in vivo would affect the immune response, BALB/c mice were primed by intramuscular injection with the DNA plasmid pCiMHGag, pCiΔG-MHGag, or pCiCAT (which expresses the irrelevant antigen chloramphenicol acetyltransferase). After 3 to 4 weeks, these mice were challenged intravenously with recombinant vaccinia virus vector encoding HIV-1 Gag. Three days later, the mice were sacrificed, and splenocytes pooled from all three mice in each group were assayed for immune responses against HIV-1 Gag (Fig. 2A). The ^{51}Cr release assay was performed by using Pvge cells as antigen-positive targets. These are derived from P815 cells but stably express HIV-1 Gag fused to EGFP (62). This assay detects *H-2*^{d}-restricted CTL activity directed at the components of HIV-1 Gag that the mice were primed against, i.e., the epitopes in HIV-1 Gag excluding p17 matrix. The bulk cytolytic activity generated in mice primed with pCiMHGag, which leads to the release of particulate Gag, was not significantly different from the response elicited in mice primed with pCiΔG-MHGag at all effector-to-target-cell ratios examined (Fig. 2B). Since the same result was obtained with P815 cells loaded with P7G peptide as targets (data not shown), peptide-loaded target cells were used in all subsequent assays of CD8$^+$-T-cell function. The CD8$^+$-T-cell response was also assayed by flow cytometric analysis of IFN-γ production following 4-h stimulation with P7G peptide (Fig. 2C and D). Although some variation in the magnitude of the response was observed, analysis of the cumulative results from six independent experiments indicated that there was no significant difference between mice primed with pCiMHGag or pCiΔG-MHGag (Fig. 2D). The IFN-γ response of CD4$^+$ T cells to overnight stimulation with recombinant HIV-1 p24 capsid protein is shown in Fig. 2E and F. There was little difference in the mean IFN-γ response of CD4$^+$ cells from mice primed with either pCiMHGag or pCiΔG-MHGag. Analysis of cumulative results from the six experiments indicated that there was insufficient statistical evidence to reject the null hypothesis of no difference between the two groups.

Antigen-specific immune responses in mice immunized with plasmid DNA expressing MHGag or ΔG-MHGag. Given previous reports of the immunogenicity of HIV-1 Gag VLPs in rodents (22, 58), it was surprising to detect no appreciable differences in the antigen-specific immune responses assayed in mice primed with pCiMHGag or pCiΔG-MHGag and later challenged with vaccinia virus expressing HIV-1 Gag. One possible explanation is that the vaccinia virus boost may have obscured any differences generated in the initial plasmid DNA prime with the different constructs. To exclude this possibility, BALB/c mice were once again immunized with pCiMHGag, pCiΔG-MHGag, or pCiCAT but boosted with repeated intramuscular DNA injections to elicit a detectable immune response (Fig. 3A). An in vivo assay was employed to detect P7G-specific CTL activity in the immunized mice (Fig. 3B). The amount of CTL killing generated in mice primed and boosted with pCiMHGag was not different from the CTL activity generated in mice immunized in the same manner with pCiΔG-MHGag. The proliferative response of splenocytes from the immunized mice to recombinant HIV-1 p24 was assayed, and the difference between pCiMHGag and pCiΔG-MHGag was, again, not statistically significant (Fig. 3C). ELISA for IFN-γ secreted into the culture supernatant was also performed in parallel and showed similar responses in mice primed with either pCiMHGag or pCiΔG-MHGag (data not shown). Finally, the HIV-1 Gag-specific antibody response in these mice was assayed by ELISA with plates coated with recombinant HIV-1 p55 Gag. No significant difference in the total HIV-1 Gag-specific IgG response was detected in mice

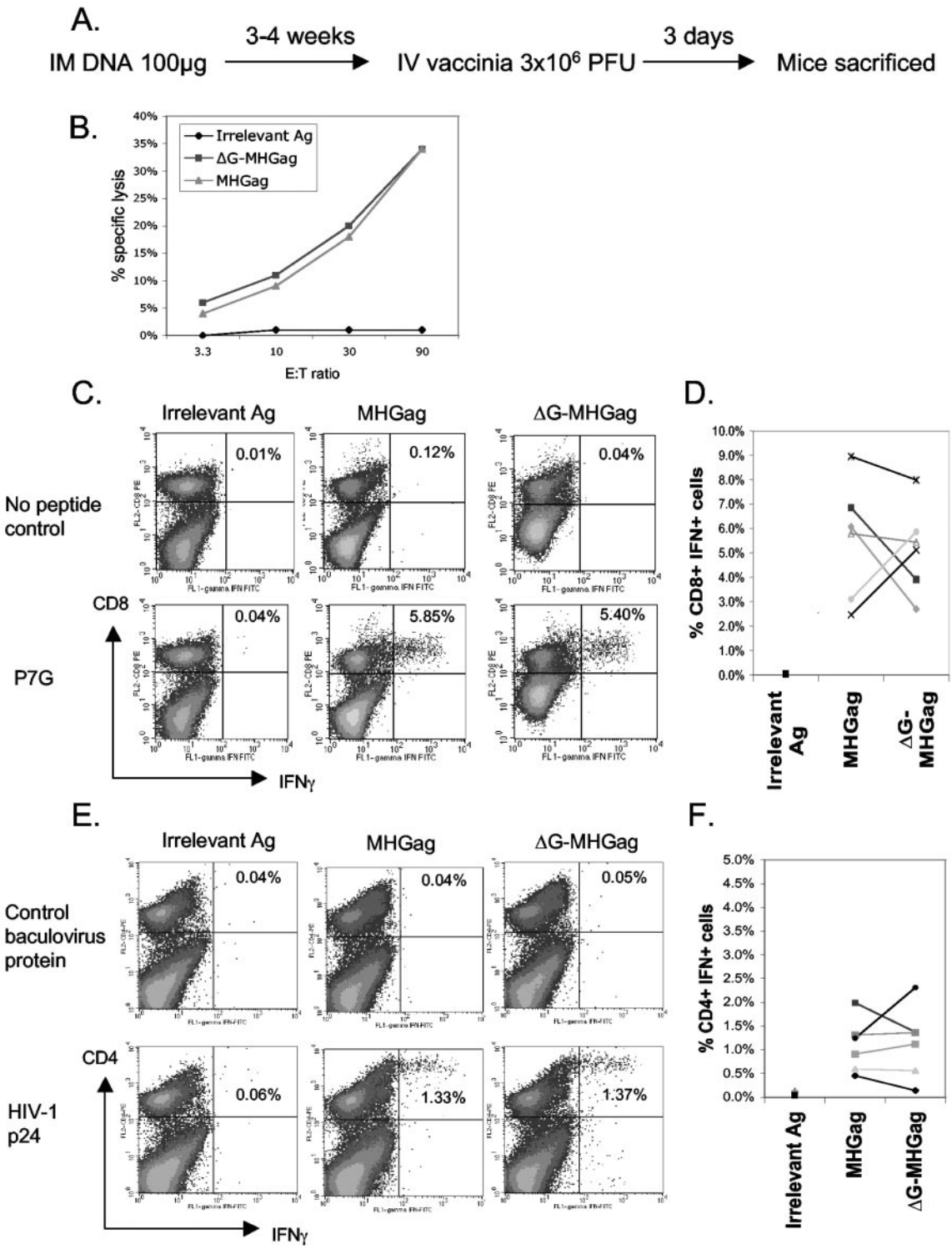
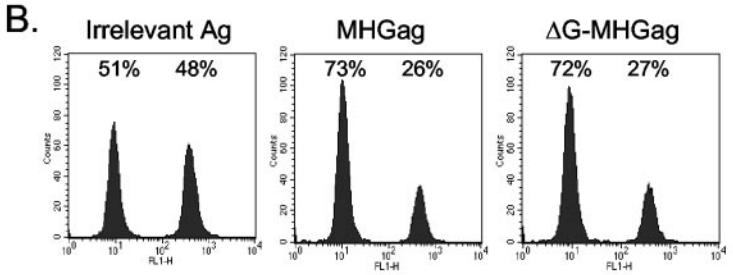
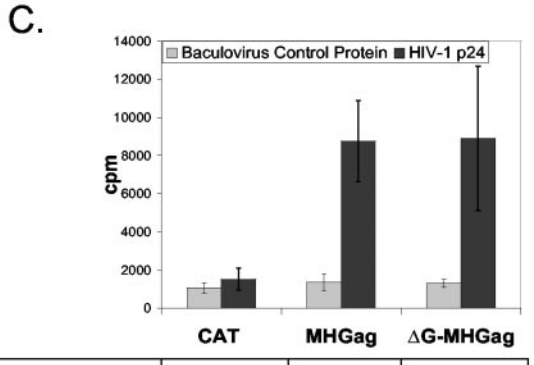


FIG. 2. T-cell response to immunization with plasmid DNA encoding MHGag or ΔG-MHGag followed by a vaccinia virus boost. (A) Immunization schedule. IM, intramuscular; IV, intravenous. (B) Chromium release assay. Negative control P815 cells or antigen (Ag)-positive Pvge cells which stably express HIV-1 Gag fused to EGFP were loaded with ^{51}Cr and used as targets in a standard 4-h chromium release assay at the indicated effector-to-target cell (E:T) ratios. The panel depicts Gag-specific lytic activity of effector splenocytes, with the background lysis of P815 cells subtracted. (C and D) Fluorescence-activated cell sorter (FACS) analysis of intracellular staining for IFN-γ production by $CD8^+$ splenocytes stimulated for 4 h with P7G peptide. The percentages indicate cells that are both $CD8^+$ and IFN-γ^+. (E and F) FACS analysis of intracellular staining for IFN-γ production by $CD4^+$ splenocytes stimulated for 15 h with recombinant HIV-1 p24 protein. The percentages indicate cells that are both $CD4^+$ and IFN-γ^+. Panels B, C, and E depict representative results from one of six independent experiments, each comprising three mice per group and three groups per experiment. The results from all six experiments are shown in panels D and F. (D) Mean MHGag, 5.54%; standard deviation, 2.41%; mean ΔG-MHGag, 5.17%; standard deviation, 1.80%. Statistical analysis by use of the unpaired *t* test generated the following result: $t(10) = 0.30$, $P = 0.77$. (F) Mean MHGag, 1.08%; standard deviation, 0.56%; mean ΔG-MHGag, 1.14%; standard deviation, 0.75%. The unpaired *t* test generated the following result: $t(10) = -0.16$, $P = 0.88$.



Comparison	Mean difference in % killing between comparison groups	95% CI	q	p value
Irrelevant Ag vs. MHGag	58%	39% to 76%	11.2	p<0.001
Irrelevant Ag vs. ΔG-MHGag	56%	38% to 74%	10.9	p<0.001
MHGag vs. ΔG-MHGag	1%	-16% to 18%	0.3	p>0.05



	CAT	MHGag	ΔG-MHGag
Stimulation index	1.44	6.42	6.86
Net cpm	462	7381	7599

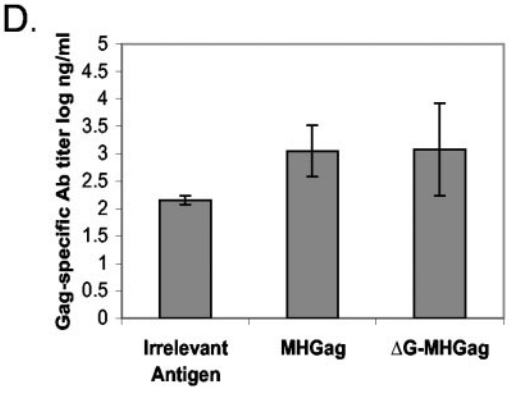


FIG. 3. Immune responses to immunization with a plasmid DNA prime-DNA boost protocol. (A) Immunization schedule. IM, intramuscular. (B) In vivo cytotoxicity assay. Splenocyte targets from syngeneic BALB/c mice were labeled with the membrane dye PKH26. Subsequently, they were either pulsed with P7G peptide and labeled with 5 μM CFSE or labeled with 0.5 μM CFSE only. High- and low-CFSE cells were mixed in equal numbers and injected intravenously into the immunized mice. Splenocytes from individual mice were recovered 15 h later and analyzed by flow cytometry for CFSE content among $PKH26^+$ cells. The histograms depict representative data from one mouse in each group. The percentages in the histogram plots indicate the remaining target cells that were either high- or low-CFSE as a fraction of total $PKH26^+$ target cells. The percent killing of peptide-loaded targets was calculated for each mouse by using the formula described in Materials and Methods. The percent killing for all of the immunized mice in each group was used to generate descriptive statistics summarizing the data for each of the three groups. Mean percent killing of peptide-loaded targets (irrelevant antigen [Ag.] group), 9%; standard deviation, 1% ($n = 7$); mean percent killing of peptide-loaded targets (MHGag), 66%; standard deviation, 19% ($n = 9$); mean percent killing of peptide-loaded targets (ΔG-MHGag), 65%; standard deviation, 14% ($n = 9$). One-way analysis of variance was subsequently used to analyze these data. Comparisons for all pairs with the Tukey-Kramer posttest generated the values which are summarized on the figure. vs., versus. (C) [^{3}H]thymidine incorporation by HIV-1 p24-specific splenocytes. Splenocytes from immunized animals were cultured for 3 days in the presence of 2.5 μg of either control baculovirus protein or recombinant HIV-1 p24/ml. During the final 12 h, the cells were incubated in the presence of 1 μCi of tritiated thymidine. Representative data from one of three independent experiments are shown. The error bars indicate the variability corresponding to ±1 standard deviation. (D) ELISA for Gag-specific antibody (Ab). Ninety-six-well flat-bottom plates were precoated with recombinant HIV-1 p55 Gag. Serial dilutions of serum samples from the immunized animals were added to the wells, and a standard curve was constructed by using commercial mouse IgG1 anti-HIV-1 p24 monoclonal antibody. Sera from 9 to 10 animals per test group were independently measured and statistically pooled to give the results depicted. The error bars represent ±1 standard deviation.

immunized with pCiMHGag compared with those immunized with pCiΔG-MHGag (Fig. 3D).

MHGag expression in primary murine cells leads to the formation of VLPs and their release into the extracellular medium. MHGag expression has been associated with the release of particulate Gag by murine cell lines in vitro. However, it was not clear whether primary murine cells would be able to do the same. While the detection of Gag antigen following sedimentation of culture supernatants through a sucrose cushion was highly suggestive of the release of particulate as opposed to soluble Gag, the nature of this particulate material had yet to be clarified. To examine this issue, we made the E1- and E3-deleted recombinant adenoviral vectors Ad5-MHGag and Ad5-G-MHGag, which expressed MHGag and ΔG-MHGag, respectively. $CD11c^+$ primary bone marrow-derived dendritic cells were generated from mice and subsequently infected with Ad5-MHGag, Ad5-ΔG-MHGag, or vAX-βgal, which expresses the irrelevant antigen β-galactosidase. Two days later, these cells were fixed, permeabilized, and stained for HIV-1 p24 (Fig. 4A). Although primary dendritic cells infected with the same MOI of either Ad5-MHGag or Ad5-ΔG-MHGag expressed similar amounts of intracellular p24, only cells infected with Ad5-MHGag released significant amounts of particulate antigen into the culture medium (Fig. 4B). Transmission EM demonstrated that the infection of dendritic cells with Ad5-MHGag was associated with the release of VLPs (Fig. 4C). These particles were circular in cross section and approximately 50 μm in diameter, possessed electron-dense cores, and morphologically resemble immature HIV-1 virions (20).

BALB/c mice were immunized by intravenous injection of dendritic cells transduced with Ad5-MHGag, Ad5-ΔG-MHGag, or vAX-EGFP. After 3 to 4 weeks, these mice received a boost of 3×10^6 PFU of recombinant vaccinia virus expressing HIV-1 Gag (Fig. 4D). The mice were sacrificed 3 days later, and the $CD8^+$-T-cell production of IFN-γ in response to stimulation with P7G peptide was assessed by flow cytometry. In spite of EM evidence to suggest that primary dendritic cells infected with Ad5-MHGag were capable of releasing VLPs into the extracellular medium, mice immunized with dendritic cells expressing MHGag demonstrated antigen-specific $CD8^+$-T-cell responses similar to mice immunized with cells expressing ΔG-MHGag (Fig. 4E).

Antigen-specific immune responses in mice immunized with adenoviral vectors expressing MHGag or ΔG-MHGag. One reason for the failure of immunization with plasmid DNA expressing MHGag to generate stronger responses than ΔG-MHGag may be the release of insufficient VLPs to make a detectable immunological difference. The transfection of cells in vivo by the intramuscular injection of plasmid DNA is not efficient. To examine the immune response primed by a vector capable of expressing large amounts of antigen in vivo, BALB/c mice were immunized intravenously with Ad5-MHGag, Ad5-ΔG-MHGag, or adenovirus expressing irrelevant antigen (Fig. 5A). Mice immunized in this manner expressed sufficient antigen to be detected by Western blot analysis of liver lysates (Fig. 5B). The antigen-specific memory response was assessed 3 weeks after priming with recombinant adenovirus. Mice immunized with Ad5-MHGag or Ad5-ΔG-MHGag showed no appreciable difference in the P7G-specific IFN-γ response of $CD8^+$ T cells as assayed by intracellular cytokine staining and flow cytometry (Fig. 5C). Little difference was also noted in the $CD4^+$-T-cell production of IFN-γ in response to stimulation with HIV-1 p24 (Fig. 5D). Thus, even when mice were immunized with a viral vaccine vector that expressed sufficient antigen to be directly detected ex vivo, antigenicity was not enhanced by VLP formation.

DISCUSSION

Intact protein antigen in particulate form is processed more efficiently by antigen-presenting cells (APCs) for major histocompatibility complex (MHC) class I-restricted presentation than the same antigen in soluble form (30). There has thus been considerable interest in the idea of using particulate Gag in the form of VLPs as a candidate AIDS vaccine. However, evaluating the immunogenicity of HIV-1 Gag VLPs in mice is complicated by the failure of HIV-1 Gag to efficiently assemble VLPs in murine cells. In this study, the problem was overcome by using an MMLV–HIV-1 chimeric Gag that releases significant amounts of VLPs when expressed in murine cells (13, 47). This has permitted an evaluation of the role played by in vivo VLP formation in the immunogenicity of HIV-1 Gag. Nevertheless, we consistently failed to detect significant differences in the ability of VLP-producing expression vectors (MHGag) to prime antigen-specific responses compared with vectors expressing the VLP-incompetent Gly mutant (ΔG-MHGag). We emphasize that these findings relate to the release of immature Gag particles that do not possess viral envelope proteins and may not apply in instances where VLPs that more closely resemble native virions are formed. We have considered several possible reasons for this lack of difference in immunogenicity. The interaction of VLPs with professional APCs is central to this discussion.

The immunogenicity of cell-exogenous antigens like VLPs is critically dependent on their ability to be taken up by APCs (23). APCs are able to process captured antigen for presentation to $CD4^+$ T cells in the context of MHC class II molecules. APCs can also process and present exogenous antigen (7, 8), including VLPs (4, 40), to MHC class I-restricted $CD8^+$ T cells via cross-priming. The particulate nature of VLPs has been considered essential to their immunogenicity. In the case of nonenveloped VLPs, the assembly of monomeric units into particles results in the formation of a high-density repetitive antigen array. Such structures may be recognized as foreign by APCs and processed efficiently for antigen presentation. Examples of viral capsomeres that can spontaneously assemble into naked icosahedral VLPs include VP60 of rabbit calicivirus (32), VP2 of Norwalk virus (5), amino-terminally truncated ORF2 of hepatitis E virus (35), parvovirus VP2 (52), papillomavirus L1 (28), and bluetongue virus VP2 (50). These form VLPs which are immunogenic without the coadministration of adjuvants.

The immunogenicity of enveloped VLPs, like those formed by HIV-1 Gag, has also been examined. HIV-1 Gag VLPs consist of a core of Gag surrounded by a lipid bilayer containing proteins derived from the producer cell (18, 43, 57). To obtain sufficient preformed VLPs for immunogenicity studies, HIV-1 Gag is often expressed in insect cells infected with recombinant baculovirus expression vectors. There is little

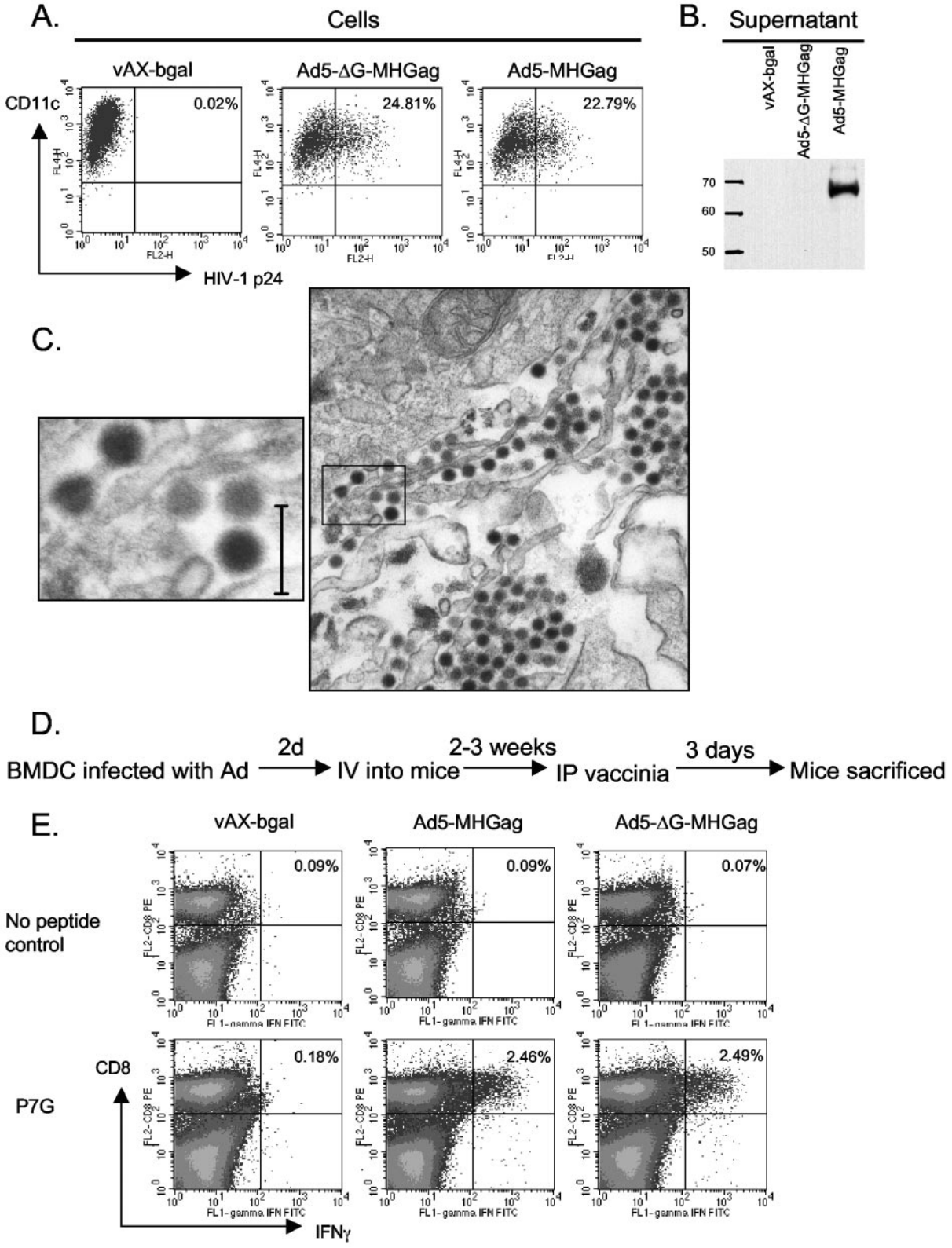


FIG. 4. Expression of MHGag and ΔG-MHGag by primary bone marrow-derived dendritic cells and the formation of VLPs. Murine CD11c$^+$ dendritic cells were purified by magnetic separation on day 6 of culture and infected with Ad5-MHGag, Ad5-ΔG-MHGag, or adenovirus encoding irrelevant antigen. (A) Flow cytometric analysis of intracellular p24 expression. Two days after infection, the dendritic cells were fixed, permeabilized, and stained with RD1-conjugated anti-HIV-1 p24 and APC-conjugated anti-CD11c. The percentages indicate p24-positive cells that are also CD11c$^+$. (B) Western analysis of ultracentrifuged culture supernatant. Four days after infection, culture supernatant from the infected cells was filtered through 0.22-μm-pore-size filters and ultracentrifuged through a 20% sucrose cushion. The pelleted material was analyzed by Western

doubt that HIV-1 Gag VLPs made in insect cells can be immunogenic (45, 59). However, the response of the immunized animal to foreign insect proteins on the VLP envelope may have affected the magnitude of the Gag-specific response.

In other attempts at examining the immunogenicity of HIV-1 Gag VLPs, mice have been immunized with plasmid DNA vectors expressing HIV-1 Gag with the intention of generating VLPs in vivo (11, 46). In contrast to Gag VLPs made by insect cells, these VLPs would be surrounded by an envelope of self proteins. However, when HIV-1 Gag is expressed in murine cells, it is misdirected into vesicular bodies in the cytoplasm and exhibits a defect in assembly at the plasma membrane. As a result, it has been estimated that the amount of HIV-1 Gag VLPs released into the extracellular medium by murine cells is reduced 500-fold (36). Therefore, it is not surprising that studies comparing the immunogenicities of wild-type HIV-1 Gag and Gag that cannot be myristoylated do not report large differences in antigen-specific responses (11, 46). The expression of either construct does not lead to the release of significant amounts of HIV-1 Gag VLPs by murine cells. In a separate report, mice were coimmunized with plasmid DNA expressing HIV-1 Gag and vesicular stomatitis virus glycoprotein G (37). The authors attributed the observed increases in immunogenicity to the in vivo release of vesicular stomatitis virus glycoprotein G-pseudotyped HIV-1 Gag VLPs by transfected cells. It may be difficult to reconcile this interpretation with the results obtained by Mariani et al. (36).

The generation of similar immune responses in mice immunized with VLP-forming MHGag or non-VLP-forming ΔG-MHGag expression vectors in this study may indicate that the processes mediating the transfer of cytosolic antigen to APCs in vivo are exquisitely efficient. If this is true, the ability of cells to release antigen in the form of VLPs may not confer significant additional benefit. One such mechanism is the release of cytosolic antigen by damaged cells. Cell damage leading to apoptotic or necrotic death has been associated with antigen presentation by APCs (1). In this report, the immunization of mice with either plasmid DNA or adenovirus expressing MHGag or ΔG-MHGag may have been associated with inflammatory cell damage leading to the release of cytosolic antigen. Immunization with the adenoviral constructs resulted in the release of sufficient antigen to be detected in serum samples by a commercial HIV-1 p24 ELISA (data not shown). However, there was no difference in the levels of p24 seen in mice given Ad5-MHGag or Ad5-ΔG-MHGag, and the signal could not be enriched by concentration across a 20% sucrose cushion (data not shown), suggesting that particulate Gag was not released in sufficient amounts to be detected by this method.

Another possibility is that the levels of chimeric Gag expression and VLP release in vivo may influence the contribution of VLPs to the immunogenicity of the construct. We attempted to address this concern by comparing mice immunized with either plasmid DNA or adenoviral expression vectors. Intramuscular immunization with plasmid DNA leads to relatively low levels of immunogen expression in vivo, and we failed to detect Gag in the serum of immunized animals, even when milligrams of plasmid was used (data not shown). Immunization with adenoviral vectors leads to high levels of antigen expression, and Gag could be readily detected in the serum of immunized animals by using a commercial ELISA for HIV-1 p24 (data not shown). Despite the differences in Gag expression elicited by these two immunization protocols in vivo, in both experiments, the ability to release VLPs did not make a significant difference in the immunogenicities of myristoylation-competent MHGag compared with myristoylation-incompetent ΔG-MHGag (Fig. 2 and 3 versus Fig. 5).

We sought to directly examine the contribution of MHGag VLPs to antigen presentation by APCs. $CD11c^+$ bone marrow-derived dendritic cells were infected ex vivo with adenoviral vectors encoding MHGag at an MOI sufficient for the transduction of 10 to 20% of cells (Fig. 4A). These cells were cultured for an additional 2 days prior to transfer into recipient mice. This allowed uninfected dendritic cells to capture and process MHGag VLPs released by the transduced cells. However, the infection of dendritic cells by adenovirus was associated with some degree of cell damage. Dendritic cells infected with Ad5-ΔG-MHGag may have released apoptotic debris that was taken up and processed by live dendritic cells in the culture. This could explain why significant differences in the $CD8^+$-T-cell response were not observed when mice were immunized with dendritic cells transduced with either MHGag or ΔG-MHGag (Fig. 4E). It is also possible that dendritic cells infected with either construct directly processed the antigen for presentation and this triggered a maximal response in the immunized mice. But we consider this explanation less likely because a relatively small number of transduced $CD11c^+$ cells ($<$2,000) was introduced into each immunized animal.

The presentation of antigen by a professional APC is necessary but may not be sufficient to initiate a robust immune response. The activation status of the APC may also be important (6). Danger signals can activate APCs (38), enhancing antigen presentation. These signals include the ligation of CD40 by CD40L and cytokines like tumor necrosis factor alpha. They also include the foreign products of microbes, e.g., lipopolysaccharide, which can activate Toll-like receptors on APCs (51). It is conceivable that the targeted delivery of VLPs to APCs and a concurrent means to activate them may enhance the immunogenicity of VLPs. These considerations are underscored by a report which identified a critical role for

blotting with polyclonal anti-HIV-1 human serum. (C) Transmission electron micrographs demonstrating MHGag VLP formation by primary murine cells. Magnification, ×52,000. Note the presence of round enveloped structures with electron-dense cores. The inset shows the highlighted area at a higher magnification. Magnification, ×155,000. Vertical bar, 100 nm. (D and E) Immunization of mice with dendritic cells expressing MHGag or ΔG-MHGag. $CD11c^+$ bone marrow-derived dendritic cells were infected with vAX-EGFP, Ad5-MHGag, or Ad5-ΔG-MHGag (Ad). Two days (2d) later, intracellular staining for HIV-1 p24 was performed on the transduced cells to ensure equivalent p24 expression prior to the intravenous (IV) injection of 10^4 dendritic cells into BALB/c mice. After 3 to 4 weeks, the response was boosted with 3×10^6 PFU of recombinant vaccinia virus expressing HIV-1 Gag. These mice were sacrificed 3 days later, and intracellular staining for IFN-γ production by $CD8^+$ T cells in response to P7G stimulation was carried out. The percentages indicate cells that are both $CD8^+$ and IFN-γ^+. Data representative of the results from three independent experiments are depicted.

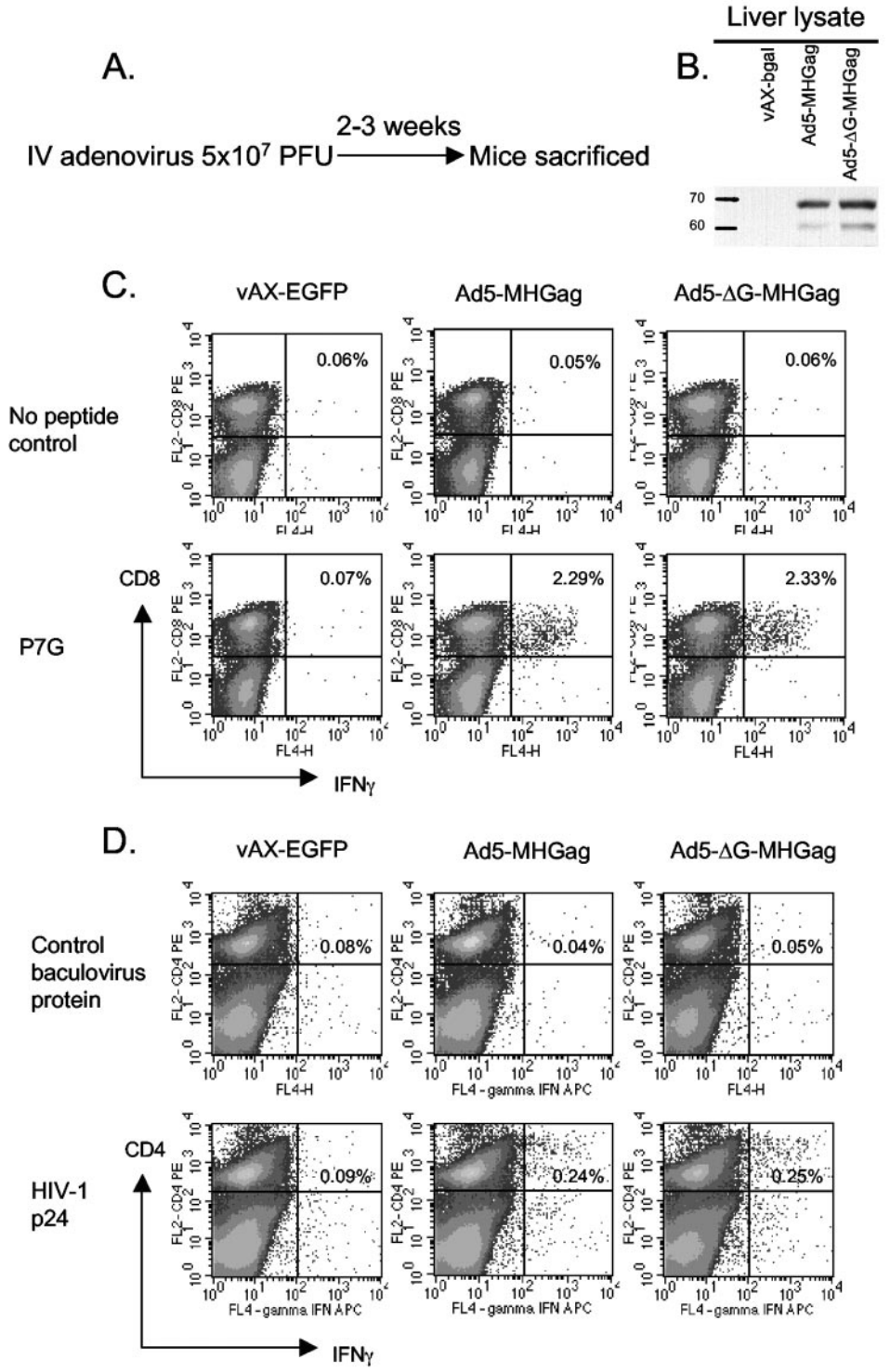


FIG. 5. T-cell response to immunization with E1- and E3-deleted adenovirus vectors encoding MHGag or ΔG-MHGag. (A) Immunization schedule. IV, intravenous. (B) Western blot of liver lysates. One animal from each group that had been immunized with Ad5-MHGag, Ad5-ΔG-MHGag, or adenovirus expressing irrelevant antigen was sacrificed on day 3 following immunization. The liver resected from each mouse was homogenized, and cell lysates were analyzed for Gag expression by immunoblotting. (C) Fluorescence-activated cell sorter (FACS) analysis of intracellular staining for IFN-γ production by $CD8^+$ splenocytes stimulated for 4 h with P7G peptide. The percentages indicate cells that are both $CD8^+$ and IFN-γ^+. (D) FACS analysis of intracellular staining for IFN-γ production by $CD4^+$ splenocytes stimulated for 15 h with recombinant HIV-1 p24 protein. The percentages indicate cells that are both $CD4^+$ and IFN-γ^+. Data representative of the results from two independent experiments are shown.

CD40-mediated APC activation in the initiation of immune responses following immunization with VLPs derived from the hepatitis B virus core antigen (55). In the case of MHGag VLPs formed in vivo, the Gag protein core is surrounded by an envelope of self lipid and proteins. These VLPs may not activate APCs due to a lack of danger signals, and APCs may not be able to distinguish these VLPs from vesicular bodies containing self antigens normally released by cells (54). This may be especially true if VLPs diffuse away from the producer cells and are taken up by distant APCs that are not affected by the inflammation that may mark the site of VLP production.

Finally, an interesting idea is that association with the plasma membrane may play an important role in the antigenicity of Gag independent of its contribution to the biogenesis of VLPs. When the matrix domain alone is expressed, VLP formation and release does not occur, even though matrix can still localize at the plasma membrane (42). It has been reported that this targeting of the plasma membrane is an important determinant of the immunogenicity of Friend murine leukemia virus matrix protein (56). This contrasts with the findings of this paper and others (11) regarding the immunogenicity of myristoylated and nonmyristoylated full-length Gag. However, differences in intracellular localization could account for the disparate observations. Full-length HIV-1 Gag, and presumably other retroviral Gag proteins, contains a nuclear export signal that counteracts the nuclear localization signal in matrix (16). This export signal is disrupted when only the matrix domain is expressed. Whereas nonmyristoylated full-length Gag remains largely cytosolic, Sugahara et al. reported that the expression of Friend murine leukemia virus matrix mutants defective for targeting of the plasma membrane was associated with nuclear sequestration of the antigen. It is unclear whether nuclear sequestration or decreased association with the plasma membrane was responsible for the effects observed. Nonetheless, it will be interesting to conduct similar experiments with HIV-1 matrix to determine whether these results can be applied to other retroviruses. It is not inconceivable that the transfer of antigen to APCs by nibbling (24) may be more efficient for cytosolic and membrane-associated antigens than for nuclear proteins.

In conclusion, we have studied the immunogenicity of an MMLV–HIV-1 Gag construct that is capable of releasing significant amounts of VLPs when expressed in primary murine cells. In this system, the ability to form morphologically immature HIV-1 Gag-derived particles that do not contain any viral envelope proteins does not appear to contribute substantially to the immunogenicity of the construct. This may have implications for the interpretation of studies testing the immunogenicity of HIV-1 Gag expression vectors in mice.

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