

# Virological and Immunological Characterization of Novel NYVAC-Based HIV/AIDS Vaccine Candidates Expressing Clade C Trimeric Soluble gp140(ZM96) and Gag(ZM96)-Pol-Nef(CN54) as Virus-Like Particles

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## ABSTRACT

The generation of vaccines against HIV/AIDS able to induce long-lasting protective immunity remains a major goal in the HIV field. The modest efficacy (31.2%) against HIV infection observed in the RV144 phase III clinical trial highlighted the need for further improvement of HIV vaccine candidates, formulation, and vaccine regimen. In this study, we have generated two novel NYVAC vectors, expressing HIV-1 clade C gp140(ZM96) (NYVAC-gp140) or Gag(ZM96)-Pol-Nef(CN54) (NYVAC-Gag-Pol-Nef), and defined their virological and immunological characteristics in cultured cells and in mice. The insertion of HIV genes does not affect the replication capacity of NYVAC recombinants in primary chicken embryo fibroblast cells, HIV sequences remain stable after multiple passages, and HIV antigens are correctly expressed and released from cells, with Env as a trimer (NYVAC-gp140), while in NYVAC-Gag-Pol-Nef-infected cells Gag-induced virus-like particles (VLPs) are abundant. Electron microscopy revealed that VLPs accumulated with time at the cell surface, with no interference with NYVAC morphogenesis. Both vectors trigger specific innate responses in human cells and show an attenuation profile in immunocompromised adult BALB/c and newborn CD1 mice after intracranial inoculation. Analysis of the immune responses elicited in mice after homologous NYVAC prime/NYVAC boost immunization shows that recombinant viruses induced polyfunctional Env-specific CD4 or Gag-specific CD8 T cell responses. Antibody responses against gp140 and p17/p24 were elicited. Our findings showed important insights into virus-host cell interactions of NYVAC vectors expressing HIV antigens, with the activation of specific immune parameters which will help to unravel potential correlates of protection against HIV in human clinical trials with these vectors.

## IMPORTANCE

We have generated two novel NYVAC-based HIV vaccine candidates expressing HIV-1 clade C trimeric soluble gp140 (ZM96) and Gag(ZM96)-Pol-Nef(CN54) as VLPs. These vectors are stable and express high levels of both HIV-1 antigens. Gag-induced VLPs do not interfere with NYVAC morphogenesis, are highly attenuated in immunocompromised and newborn mice after intracranial inoculation, trigger specific innate immune responses in human cells, and activate T (Env-specific CD4 and Gag-specific CD8) and B cell immune responses to the HIV antigens, leading to high antibody titers against gp140. For these reasons, these vectors can be considered vaccine candidates against HIV/AIDS and currently are being tested in macaques and humans.

The demand for an effective HIV vaccine capable of inducing long-lasting protective immunity has stimulated the development of recombinant live vaccine candidates exerting good safety and immunogenicity profiles. The Thai phase III clinical trial (RV144), in which the recombinant canarypox virus vector ALVAC and the bivalent HIV-1 protein gp120 B/E in alum used in a prime-boost strategy showed a modest 31.2% protective efficacy against HIV infection (1), has increased interest in the use of improved attenuated poxvirus vectors as HIV vaccine candidates.

Among poxviruses, the highly attenuated vaccinia virus (VACV) strain NYVAC is being evaluated in both preclinical and clinical trials as a vaccine against several emergent infectious diseases and cancer (2, 3). The NYVAC (vP866) strain was derived from a plaque-purified isolate (VC-2) of the Copenhagen VACV strain (VACV-COP) after the precise deletion of 18 open reading frames (ORFs) implicated in pathogenesis, virulence, and host

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range functions (4). Despite its restricted replication in human and most mammalian cell types, NYVAC provides high levels of heterologous gene expression and elicits antigen-specific immune responses in animals and humans (2, 3, 5–7). However, the limited immunogenicity elicited in clinical trials by attenuated poxvirus vectors expressing HIV antigens (3), like modified vaccinia virus Ankara (MVA), NYVAC, and canarypox and fowlpox viruses, together with the modest efficacy (31.2%) against HIV infection of the canarypox ALVAC vector with HIV-1 gp120 protein, which was obtained in the RV144 phase III clinical trial (1), emphasized the urgent requirement of novel optimized poxvirus-based HIV vaccine vectors with improved antigen presentation and immunogenicity profiles.

With regard to attenuated poxvirus vectors, different strategies have been addressed to enhance their immunogenicity, like the use of costimulatory molecules, the combination of heterologous vectors, the improvement of virus promoter strength, the enhancing of vector replication capacity, the combined use of adjuvants, and the deletion of immunomodulatory viral genes still present in the viral genome (3, 8). The latter strategy already has been pursued in the context of MVA and NYVAC genomes. A number of MVA deletion mutants lacking VACV immunomodulators have been generated to date and tested in mice (9–15) and macaques (16, 17), showing an enhancement in the overall immune responses to HIV-1 antigens. Similarly, NYVAC vectors with single or double deletions in VACV genes *B19R* and *B8R*, encoding type I and type II interferon (IFN) binding proteins, respectively (18), or with a single deletion in the inhibitor of toll-like receptor (TLR) signaling, *A46R* (19), increased the immune responses to HIV antigens in the mouse model.

Here, we describe a different strategy to enhance the immune responses triggered by an NYVAC-based vector against HIV-1 antigens. This strategy is not based on the modification of the vector backbone itself but in the insertion of novel optimized HIV-1 antigens. To date, NYVAC-based HIV vaccine candidates have been designed in a manner to express both Env and Gag-Pol-Nef (GPN) antigens from the same viral locus. In this context, NYVAC-C (vP2010), a recombinant vector expressing clade C 97CN54 HIV-1 gp120 and Gag-Pol-Nef proteins from the thymidine kinase (TK) locus, has been tested in a phase I clinical trial (EV01) in healthy, HIV-negative volunteers, showing a good safety profile and triggering T cell immune responses against HIV-1 antigens in 50% of the vaccinees assessed, with responses to Env representing the majority of the total responses (20). Another phase I clinical trial (EV02) was performed to compare the safety and immunogenicity of a DNA-C prime/NYVAC-C boost regimen to that of NYVAC-C alone (21). This trial showed the good safety profile of both vaccines and revealed that a DNA prime enhances the HIV-1-specific T and B cell immune responses (21). As previously reported in the EV01 clinical trial, responses were directed against Env in the majority of the volunteers (21), and these responses were polyfunctional and long-lasting (22). A similar NYVAC-based vaccine candidate expressing Env and Gag-Pol-Nef antigens but from HIV-1 clade B (NYVAC-B) also has been evaluated in a phase I clinical trial (Theravac-01) in HIV-infected patients successfully treated with antiretroviral therapy (23). NYVAC-B was safe and highly immunogenic, triggering both an expansion of preexisting T cell immune responses and the appearance of newly detected HIV-specific CD4 and CD8 T cell

responses (23). Moreover, immunization mostly induced an increase in polyfunctional Gag-specific T cell responses (23).

While in the previous clinical trials with NYVAC vectors the Env protein was produced as a monomer of gp120 and Gag-Pol-Nef as a fusion polyprotein unable to form VLPs, with both HIV antigens being synthesized at the same time within the infected cell and with immunization with NYVAC vector alone being about half that with a heterologous DNA prime/NYVAC boost, we reasoned that generating novel NYVAC vectors expressing higher levels of HIV antigens and independently producing trimeric forms of Env and Gag particles as VLPs could be a more effective approach to favor both B and T cell immune responses. DNA constructs producing a soluble trimeric gp140 protein and a Gag-Pol-Nef fusion protein in a configuration that has been shown to allow VLP formation recently have been reported to lead to overall higher expression levels and enhanced immunogenicity with the analogous CN54-derived antigens after DNA vaccination in the BALB/c mouse model (24).

In the present study, we describe the generation and virological and immunological characterization of two novel NYVAC-based HIV vaccine candidates expressing gp140 from HIV-1 clade C 96ZM651 (here termed ZM96; NYVAC-gp140) or HIV-1 clade C Gag(ZM96)-Pol-Nef(CN54) (NYVAC-Gag-Pol-Nef). The HIV-1 antigens have been designed and synthesized to produce a soluble trimeric gp140 protein and a Gag-Pol-Nef protein that is processed into the expression of the 55-kDa Gag protein that is able to induce VLPs. The virological, biochemical, and cell characteristics of these vectors, as well as their morphogenesis, safety, and immunogenicity, have been assessed in cultured cells and in mice.

## MATERIALS AND METHODS

**Ethics statement.** The animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA) of Centro Nacional de Biotecnología (CNB-CSIC; Madrid, Spain) in accordance with national and international guidelines and with the Royal Decree (RD 1201/2005) (permit numbers 152/07 and 080030).

**Cells and viruses.** African green monkey kidney cells (BSC-40), human cells (HeLa), and primary chicken embryo fibroblast (CEF) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% newborn calf serum (NCS) for BSC-40 and HeLa cells or 10% fetal calf serum (FCS) for CEF cells. The THP-1 human monocytic cell line was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen), and 10% FCS. THP-1 cells were differentiated into macrophages by treatment with 0.5 mM phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 24 h before use. Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors (Blood Center, Lausanne, Switzerland) were purified by Ficoll-Hypaque density gradient (GE Healthcare), and human primary monocytes (>97% purity) were isolated using anti-CD14 beads (Miltenyi Biotec). Monocytes were cultured in RPMI containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, 50 µM 2-mercaptoethanol (all from Invitrogen), and 10% FCS. Cells were maintained in a humidified air-5% CO<sub>2</sub> atmosphere at 37°C. The poxvirus strains used in this work included Western Reserve (WR), Copenhagen VC-2 strain (Cop; provided by Bertram Jacobs), the genetically attenuated VACV-based vector NYVAC-WT (vP866; provided by Sanofi-Pasteur), used as the parental vector for the generation of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses, and the recombinant NYVAC-C expressing gp120 as a cell-released product and Gag-Pol-Nef as an intracellular polyprotein from the clade C CN54 HIV-1 isolate (25). Virus infections were performed with 2% NCS or FCS. All viruses were grown in primary CEF cells and similarly purified through

two 36% (wt/vol) sucrose cushions, and the virus titers were determined by immunostaining plaque assay in BSC-40 cells as previously described (26). The titer determinations of the different viruses were performed at least three times.

**Construction of plasmid transfer vectors pLZAW1-gp140(ZM96) and pLZAW1-Gag(ZM96)-Pol-Nef(CN54).** The plasmid transfer vectors pLZAW1-gp140(ZM96) and pLZAW1-Gag(ZM96)-Pol-Nef(CN54), used for the construction of the recombinant viruses NYVAC-gp140(ZM96), expressing the HIV-1 clade C ZM96 gp140 gene, and NYVAC-Gag(ZM96)-Pol-Nef(CN54), expressing the HIV-1 clade C Gag(ZM96)-Pol-Nef(CN54) gene, respectively, were provided by Ralf Wagner (University of Regensburg). The gp140 gene was derived from the Env gene of the clade C 96ZM651 HIV-1 isolate (accession number AF286224), containing the autologous signal peptide, a mutated cleavage site at the gp120-gp41 boundary (REKR to REKS), and the extracellular part of gp41 until amino acid 673 of Env. The gp140 DNA sequence was synthesized with optimization for human expression and cloned into pLZAW1 plasmid to generate the plasmid transfer vector pLZAW1-gp140(ZM96). The Gag-Pol-Nef sequence consists of (i) the entire Gag gene from the clade C ZM96 HIV-1 isolate, also optimized for human expression, except for the region harboring the ribosomal frameshift site (from nucleotide 1279 to the stop codon), followed in the *trans*-frame after p6\* by (ii) the Pol-Nef cassette derived from the clade C 97CN54 HIV-1 isolate (accession number AX149647.1) with the modifications described in reference 27. The Gag-Pol-Nef DNA sequence also was cloned into pLZAW1 to generate the plasmid transfer vector pLZAW1-Gag(ZM96)-Pol-Nef(CN54). Both plasmids are designed for a blue/white plaque screening. They contain thymidine kinase (TK) left and right flanking sequences, a short TK left-arm repeat, a VACV *E3L* promoter driving the  $\beta$ -galactosidase ( $\beta$ -gal) expression cassette, and the ampicillin resistance gene. Between the two TK flanking sequences there is a VACV synthetic early/late (E/L) promoter driving the HIV-1 genes. Both plasmid transfer vectors direct the insertion of gp140 or Gag-Pol-Nef genes into the TK locus of the NYVAC genome.

**Construction of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses.** A total of  $3 \times 10^6$  BSC-40 cells were infected with NYVAC-WT at a multiplicity of infection (MOI) of 0.005 PFU/cell and transfected 1 h later with 6  $\mu$ g DNA of pLZAW1-gp140(ZM96) or pLZAW1-Gag(ZM96)-Pol-Nef(CN54) using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. After 72 h postinfection (hpi), the cells were harvested, lysed by freeze-thaw cycling, sonicated, and used for recombinant virus screening. Recombinant NYVAC viruses containing gp140 or Gag-Pol-Nef genes and transiently coexpressing the  $\beta$ -gal marker gene were selected by 4 consecutive rounds of plaque purification in BSC-40 cells stained with 5-bromo-4-chloro-3-indolyl  $\beta$ -galactoside (1.2 mg/ml). After the desired recombinant viruses were isolated by screening for the expression of  $\beta$ -gal activity, further propagation of the recombinant viruses led to the self-deletion of  $\beta$ -gal by homologous recombination between the TK left arm and the short TK left-arm repeat that are flanking the marker. Thus, in the following 3 rounds, recombinant NYVAC viruses containing gp140 or Gag-Pol-Nef genes and having deleted the  $\beta$ -gal marker gene were isolated by plaque purification screening for nonstaining viral foci in BSC-40 cells in the presence of 5-bromo-4-chloro-3-indolyl  $\beta$ -galactoside (1.2 mg/ml). The resulting NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses were expanded first in BSC-40 and finally in CEF cells, and the crude preparations obtained were used for the propagation of both viruses in large cultures of CEF cells followed by virus purification through two 36% (wt/vol) sucrose cushions and titrated by immunostaining plaque assay in BSC-40 cells.

**PCR analysis of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses.** To test the identity and purity of both recombinant viruses, viral DNA was extracted from BSC-40 cells infected at 5 PFU/cell with NYVAC-WT, NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef(CN54) for 24 h. Cell membranes were dis-

rupted using sodium dodecyl sulfate (SDS) followed by proteinase K treatment (0.2 mg/ml proteinase K in 50 mM Tris-HCl, pH 8, 100 mM EDTA, pH 8, 100 mM NaCl, and 1% SDS for 1 h at 55°C) and phenol extraction of viral DNA. Primers TK-L (5'-TGATTAGTTTGTATGCGA TTC-3') and TK-R (5'-CTGCCGTATCAAGGACA-3'), spanning TK flanking regions, were used for PCR analysis of the TK locus. The amplification reactions were carried out with Platinum *Taq* DNA polymerase (Invitrogen) according to the manufacturer's recommendations. The size of the inserts is shown in Fig. 1A.

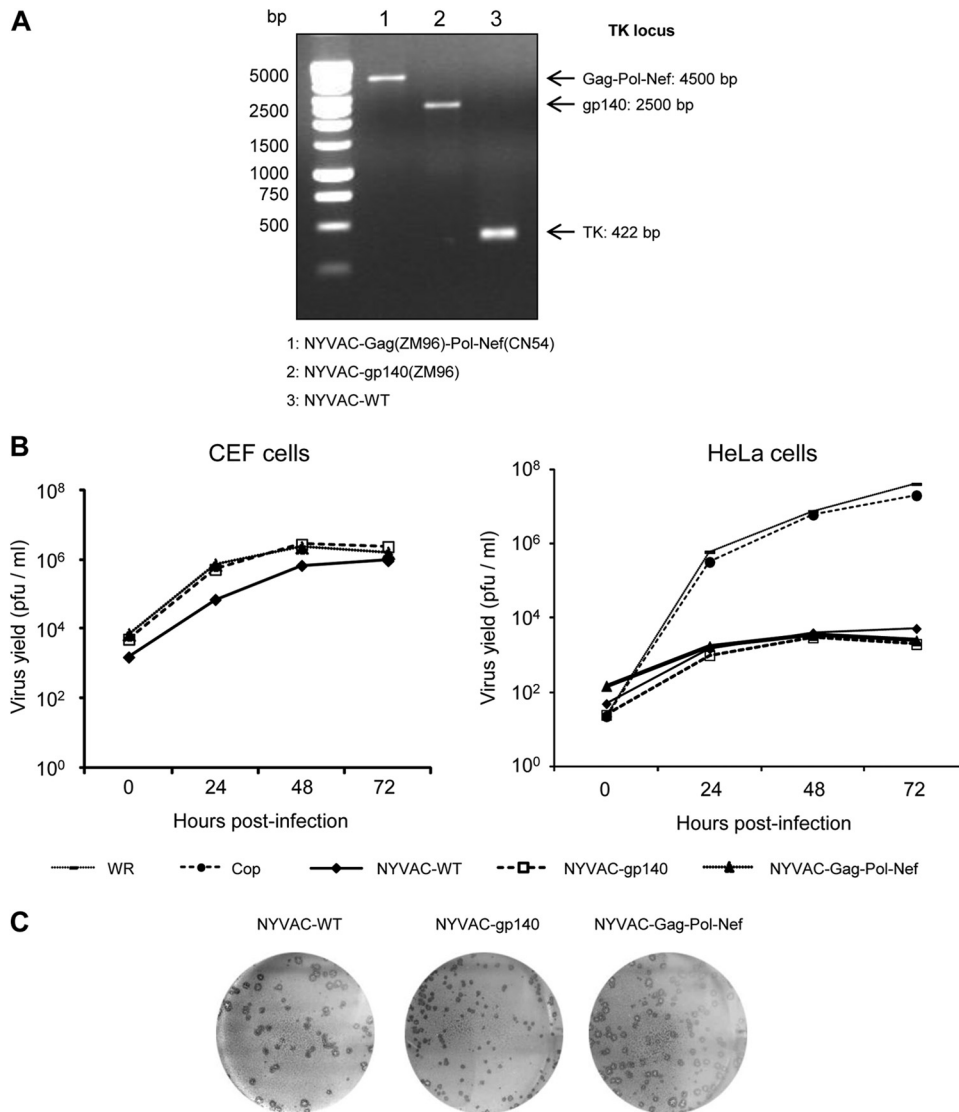
**Analysis of virus growth.** To determine virus growth profiles, monolayers of CEF (permissive) or HeLa (nonpermissive) cells grown in 12-well plates were infected in duplicate at 0.01 PFU/cell with WR, Cop, NYVAC-WT, NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef(CN54). Following virus adsorption for 60 min at 37°C, the inoculum was removed and the infected cells were incubated with fresh DMEM containing 2% FCS or NCS at 37°C in a 5% CO<sub>2</sub> atmosphere. At different times postinfection (0, 24, 48, and 72 h), cells were harvested by scraping (lysates at  $5 \times 10^5$  cells/ml), freeze-thawed three times, and briefly sonicated. Virus titers in cell lysates were determined by immunostaining plaque assay in BSC-40 cells using rabbit polyclonal anti-VACV strain WR (1:1,000; CNB), followed by anti-rabbit-horseradish peroxidase (HRP) (1:1,000; Sigma).

**Plaque size.** To determine the plaque size of both recombinant viruses, BSC-40 cells grown in 6-well plates were infected with serial dilutions of NYVAC-WT, NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef(CN54). At 48 hpi, cells were fixed and incubated with rabbit polyclonal anti-VACV strain WR (1:1,000; CNB), followed by anti-rabbit-HRP (1:1,000; Sigma).

**Time course expression of HIV-1 proteins gp140 and Gag-Pol-Nef by Western blot analysis.** To test the correct expression of HIV-1 antigens by both recombinant viruses, a time course analysis was performed. Monolayers of BSC-40 cells grown in 24-well plates were mock-infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C (used as a control for gp120 and nonprocessed Gag-Pol-Nef antigens), NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef(CN54). At different times postinfection (2, 6, 16, and 24 h), infected cells were collected. Cells extracts were fractionated by 8% SDS-PAGE and analyzed by Western blotting using polyclonal anti-gp120 antibody (1:3,000; CNB) or polyclonal anti-gag p24 serum (1:1,000; ARP 432; NIBSC, Centralised Facility for AIDS Reagents) to evaluate the expression of gp140 or Gag-Pol-Nef proteins, respectively. Anti-rabbit HRP (1:5,000; Sigma) was used as a secondary antibody. The immunocomplexes were detected by enhanced chemiluminescence (ECL; GE Healthcare).

**Detection of HIV-1 proteins gp140 and Gag-Pol-Nef in the supernatant of infected cells by Western blot analysis.** To determine the presence of HIV-1 antigens gp140 and Gag-Pol-Nef in the supernatant of infected cells, monolayers of BSC-40 cells grown in 6-well plates were infected at 5 PFU/cell with NYVAC-WT, NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef(CN54). At different times postinfection (6, 18, and 24 h), cells were collected and centrifuged at 3,000 rpm for 5 min. The supernatant (S) was removed and treated with 10% trichloroacetic acid (TCA) for 1 h on ice to precipitate the proteins and then centrifuged at 3,000 rpm for 5 min. The supernatant from this centrifugation step was discarded, and the pellet was washed with acetone, centrifuged at 3,000 rpm for 5 min, and resuspended in Laemmli buffer with 2-mercaptoethanol. Cellular pellets (P) were lysed in cold buffer (50 mM Tris-HCl, pH 8, 0.5 M NaCl, 10% NP-40, 1% SDS) for 30 min on ice and centrifuged at 4°C and 13,000 rpm for 20 min. The supernatant was transferred to a new tube and mixed with 2 $\times$  Laemmli buffer with 2-mercaptoethanol. The supernatant and pellet samples were fractionated by 8% SDS-PAGE and analyzed by Western blotting using the rabbit polyclonal anti-gp120 antibody (1:3,000; CNB) or the rabbit polyclonal anti-gag p24 serum (1:1,000; ARP 432; NIBSC, Centralised Facility for AIDS Reagents, United Kingdom) to evaluate the expression of gp140 or Gag-Pol-Nef proteins, respectively. The anti-rabbit HRP (1:5,000; Sigma) was used as a secondary antibody. The immunocomplexes were detected by ECL (GE Healthcare).





**FIG 1** *In vitro* characterization of NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses. (A) Confirmation of HIV-1 antigen insertion by PCR analysis. Viral DNA was extracted from BSC-40 cells infected with NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef at 5 PFU/cell. Primers TK-L and TK-R, spanning *J2R* (TK) flanking sequences, were used for PCR analysis of the TK locus. In parental NYVAC, a 422-bp product is obtained, while in NYVAC-gp140 or NYVAC-Gag-Pol-Nef a unique 2,500- or 4,500-bp product is observed. (B) Analysis of virus growth in permissive and nonpermissive cell lines. Monolayers of CEF (permissive) or HeLa (nonpermissive) cells were infected with WR, Cop, NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef at 0.01 PFU/cell. At different times postinfection (0, 24, 48, and 72 h), cells were collected and infectious viruses were quantified by immunostaining plaque assay in BSC-40 cells. (C) Analysis of the plaque size. BSC-40 cells were infected with serial dilutions of NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef. At 48 hpi, cells were fixed and incubated with rabbit polyclonal anti-VACV strain WR followed by anti-rabbit HRP.

To detect the presence of the trimeric form of gp140 in the supernatant of infected cells, monolayers of BSC-40 cells grown in 24-well plates were infected at 2 PFU/cell with NYVAC-WT, NYVAC-C, or NYVAC-gp140(ZM96). At different times postinfection (6 and 24 h), cells were collected and centrifuged at 3,000 rpm for 5 min. The supernatants were harvested and treated as described above. The resulting pellet samples were resuspended in Laemmli buffer without 2-mercaptoethanol, fractionated by 8% SDS-PAGE, and analyzed by Western blotting using the rabbit polyclonal anti-gp120 antibody.

**Expression of HIV-1 proteins gp140 and Gag-Pol-Nef by confocal microscopy analysis.** For the detection of HIV-1 proteins expressed by both NYVAC-based recombinant viruses, BSC-40 cells cultured on glass coverslips at a confluence of 50% were mock infected or infected with NYVAC-gp140(ZM96) or NYVAC-Gag(ZM96)-Pol-Nef(CN54) at 0.5

PFU/cell. At 6 or 24 h postinfection, cells were washed with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde (PFA) in PBS at room temperature for 15 min. After fixation, coverslips were washed with PBS three times, quenched for 15 min in the presence of 50 mM  $\text{NH}_4\text{Cl}$ , and permeabilized with 0.05% saponin in PBS, and nonspecific unions with antibodies were blocked with 5% FCS. Rabbit polyclonal anti-gp120 antibody (diluted 1:250 in 0.05% saponin–5% FCS; CNB) or rabbit polyclonal anti-gag p24 serum (diluted 1:500 in 0.05% saponin–5% FCS; ARP 432; NIBSC, Centralised Facility for AIDS Reagents, United Kingdom) was used to stain gp140 or Gag protein expressed by BSC-40 cells infected with NYVAC-gp140(ZM96) or NYVAC-Gag(ZM96)-Pol-Nef(CN54), respectively, for 1 h at room temperature. In addition, mouse monoclonal anti-protein disulfide isomerase (PDI) (1:200; Bionova) was used to stain the endoplasmic reticulum (ER) for 1 h at room temperature.

After incubation with the different primary antibodies, coverslips were washed with PBS three times and incubated with specific secondary rabbit or mouse antibodies conjugated with fluorochromes for 1 h in the dark at room temperature. Goat anti-rabbit Alexa 488 (green staining; Life Technologies) or goat anti-mouse Alexa 594 (red staining; Life Technologies) were used at a dilution of 1:500 in 0.05% saponin–5% FCS. Cells then were washed three times with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; 1:200; Sigma) to detect cell nuclei and phalloidin (1:500; Sigma) or wheat germ agglutinin (WGA; 1:250; Life Technologies) to detect actin or *trans*-Golgi membranes, respectively. Coverslips then were washed with PBS and mounted on glass slides in the presence of Pro-Long Gold antifade reagent (Invitrogen). Optical sections of the cells were acquired using a Leica TCS SP5 microscope, and images were recorded and processed by the specialized software LasAF (Leica Microsystems).

**Detection of Gag-induced VLPs by EM analysis.** For the visualization by electron microscopy (EM) of the singular structures produced by NYVAC-Gag(ZM96)-Pol-Nef(CN54) viral infection, confluent BSC-40 cells grown on 60-cm<sup>2</sup> plastic culture dishes were infected with NYVAC-Gag(ZM96)-Pol-Nef(CN54) at 2.5 PFU/cell. At 6 or 18 h postinfection, cell monolayers were fixed *in situ* for 1 h at room temperature with 2% glutaraldehyde–1% tannic acid in 0.4 M HEPES buffer, pH 7.2. Fixed monolayers then were carefully scraped from the plastic dish and transferred to Eppendorf tubes in the fixative solution. Cells were centrifuged to discard the fixative and maintained in HEPES buffer at 4°C until processing by conventional embedding in the epoxy resin EML-812 (Taab Laboratories, Adermaston, Berkshire, United Kingdom). Cells then were postfixed in a mixture of 1% osmium tetroxide–0.8% potassium ferricyanide in distilled water for 1 h at 4°C, subsequently washed with HEPES buffer, and stained with 2% uranyl acetate. After extensive washes with HEPES buffer, the samples were dehydrated in increasing concentrations of acetone (50, 70, 90, and 100%) for 15 min each at 4°C. Cells then were infiltrated in the epoxy resin at room temperature for 1 day and maintained at 60°C for 3 days to allow resin polymerization. Ultrathin sections (70 nm thick) of the samples were sliced using a Leica EM UC6 ultramicrotome and collected on copper grids. The cell sections finally were stained with saturated uranyl acetate and lead citrate by ordinary procedures. Sections of NYVAC-Gag(ZM96)-Pol-Nef(CN54)-infected cells were analyzed in a JEOL 1011 transmission electron microscope operating at 100 kV. Digital images were recorded with an ES1000W Erlangshen charge-coupled device (CCD) camera (Gatan, California, USA).

**Analysis of innate immune response by quantitative real-time PCR (RT-PCR).** Total RNA was isolated from THP-1 cells mock infected or infected with NYVAC-WT, NYVAC-C, NYVAC-gp140, or NYVAC-Gag-Pol-Nef (3 or 6 h; 5 PFU/cell) with the RNeasy kit (Qiagen). Reverse transcription of at least 500 ng of RNA was performed with the QuantiTect reverse transcription kit (Qiagen). Quantitative PCR was performed with a 7500 real-time PCR system (Applied Biosystems) and power SYBR green PCR master mix (Applied Biosystems). The expression levels of the genes for IFN- $\beta$ , IFIT2, MDA-5, and hypoxanthine phosphoribosyltransferase (HPRT) were analyzed by real-time PCR with specific oligonucleotides (sequences are available upon request). Specific gene expression was expressed relative to the expression of the gene for HPRT in arbitrary units. All samples were tested in duplicate, and two different experiments were performed with each cell type.

**Analysis of innate immune response by Luminex technology.** Human monocytes were plated in a 96-well plate ( $2 \times 10^5$  cells/well), and after 24 h of resting, medium was changed and cells were mock infected or infected for 24 h with NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef at 1 PFU/cell. The production of macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), MIP-1 $\beta$ , IFN- $\gamma$ -inducible protein 10 (IP-10), RANTES, GRO $\alpha$  (chemokine [C-X-C motif] ligand 1 [CXCL1]), and monocyte chemoattractant protein 1 (MCP-1) in the supernatant of infected cells was measured with the ProcartaPlex human cytokine and chemokine assay (eBioscience) using the Luminex technology.

**Assessment of attenuation of NYVAC-based vectors. (i) Immuno-compromised adult mice.** To determine the attenuation profile of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54), 6- to 8-week-old female BALB/c mice purchased from Harlan Laboratories were cyclophosphamide treated (six animals per group) with a first dose of 300 mg of cyclophosphamide (Sigma)/kg of body weight administered intraperitoneally (i.p.) and with a second dose of 150 mg/kg of the drug 5 days later (28). Twenty-four hours after cyclophosphamide treatment, drug-treated and nontreated control animals were mock infected with PBS or infected intracranially (i.c.) with  $10^6$ ,  $10^7$ , or  $10^8$  PFU of the NYVAC recombinant viruses per animal or with  $10^5$ ,  $10^6$ , or  $10^7$  PFU of wild-type Copenhagen strain per animal. For the intracranial infections, mice were anesthetized with isoflurane, and inoculations with the different viruses were performed approximately half way between the eye and ear and just off the midline using a 30-gauge needle (BD Biosciences) with a total volume of 30  $\mu$ l, as previously described (29, 30). Loss of weight and other signs of illness (absence of grooming, rough coat hair, loss of mobility, inflammation of the eye membrane, and hunched posture) were monitored daily for up to 30 days postinoculation. Animals that lost 25% or more of their initial weight were euthanized in a CO<sub>2</sub> chamber.

**(ii) Newborn mice.** Pregnant CD1 mice were purchased from Charles River at 10 days of gestation. The animals were housed one animal per cage. Intracranial infections with different doses of Cop, NYVAC-C, NYVAC-gp140(ZM96), or NYVAC-Gag(ZM96)-Pol-Nef (CN54) virus, using a total volume of 10  $\mu$ l, were conducted at 48 to 72 h postbirth of the pups (10 pups per virus), using a 27-gauge needle, as previously described (31). Animals were monitored twice daily for 14 days for morbidity and mortality, as previously described (32).

**Peptides.** The HIV-1 ZM96 gp140 peptides were provided by the Centralised Facility for AIDS Reagents, NIBSC, United Kingdom. They spanned the HIV-1 gp140 from clade C (ZM96) included in the recombinant virus NYVAC-gp140(ZM96) as consecutive 15-mers overlapping by 11 amino acids. They were pooled in three different Env peptide pools: Env-1 (61 peptides), Env-2 (65 peptides), and Env-3 (40 peptides). The HIV-1 Gag(ZM96) peptide (sequence, AMQMLKDTI) was previously described as an H-2<sup>d</sup>-restricted cytotoxic T-lymphocyte (CTL) epitope (33) and was provided by the Proteomic Service at the CNB-CSIC, Spain. The HIV-1 peptide Pol-1 (sequence, LVGPT-PVNI; CNB) also was previously described as an H-2<sup>d</sup>-restricted CTL epitope (27).

**Mouse immunization schedule.** BALB/c mice (6 to 8 weeks old) were purchased from Harlan Laboratories. For the homologous NYVAC prime/NYVAC boost immunization protocol performed to assay the immunogenicity of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54), groups of animals ( $n = 4$ ) were immunized with  $2 \times 10^7$  PFU of NYVAC-WT, NYVAC-gp140(ZM96), NYVAC-Gag(ZM96)-Pol-Nef(CN54), or NYVAC-MIX [ $1 \times 10^7$  PFU of NYVAC-gp140(ZM96) plus  $1 \times 10^7$  PFU of NYVAC-Gag(ZM96)-Pol-Nef(CN54)] by the i.p. route. Two weeks later, animals were immunized as in the priming. Ten days after the last immunization, mice were sacrificed and spleens processed for intracellular cytokine staining (ICS) assay, and sera were harvested for enzyme-linked immunosorbent assay (ELISA) to measure the cellular and humoral adaptive immune response against HIV-1 antigens, respectively. Two independent experiments were performed.

**ICS assay.** After an overnight rest,  $4 \times 10^6$  splenocytes from immunized mice (depleted of red blood cells) were seeded on 96-well plates and stimulated during 6 h in complete RPMI 1640 media supplemented with 10% FCS containing 1  $\mu$ l/ml GolgiPlug (BD Biosciences), anti-CD107a-Alexa 488 (BD Biosciences), and 5  $\mu$ g/ml of the different HIV-1 peptide pools (Env-1, Env-2, and Env-3) or 10  $\mu$ g/ml of the different HIV-1 peptides (Gag and Pol-1). At the end of the stimulation period, cells were washed, stained for the surface markers, fixed, and permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and stained intracellularly using the appropriate fluorochromes. Dead cells were excluded from the analysis using the violet LIVE/DEAD stain kit (Invitrogen). For functional analysis

ses, the following fluorochrome-conjugated antibodies were used: CD3-phycoerythrin (PE)-CF594, CD4-allophycocyanin (APC)-Cy7, CD8-V500, IFN- $\gamma$ -PE-Cy7, interleukin-2 (IL-2)-APC, and tumor necrosis factor alpha (TNF- $\alpha$ )-PE (all from BD Biosciences). Cells were acquired using a GALLIOS flow cytometer (Beckman Coulter). Analyses of the data were performed using FlowJo software, version 8.5.3 (Tree Star, Ashland, OR). The number of lymphocyte-gated events ranged between  $1 \times 10^5$  and  $1 \times 10^6$ . After gating, Boolean combinations of single functional gates were created using FlowJo software to determine the frequency of each response based on all possible combinations of cytokine expression or all possible combinations of differentiation marker expression. For each population, background responses detected in the nonstimulated control samples were subtracted from those detected in stimulated samples for every specific functional combination, and the percentages of cells producing cytokines obtained in the NYVAC control populations also were subtracted from all groups in order to remove the nonspecific responses detected as the background.

**Antibody measurement by ELISA.** Binding antibodies to gp140 and p17/p24 proteins in serum from immunized mice were determined by ELISA as previously described (25). Serum samples from naive and immunized mice were serially diluted 3-fold (gp140) or 2-fold (p17/p24) in duplicate and reacted against 0.9  $\mu$ g/ml of the recombinant CN54gp140 purified protein (provided by Greg Spies, Fred Hutchinson Cancer Research Center, Seattle, WA) or 1  $\mu$ g/ml of the recombinant p17/p24 protein (C-clade consensus sequence; ARP695.2; NIBSC, Centralised Facility for AIDS Reagents, United Kingdom). The antibody titers of gp140- or p17/p24-specific total IgG were defined as the last dilution of serum that resulted in 3 times the mean optical density at 450 nm of the naive control.

**Data analysis and statistics.** For the statistical analysis of ICS data, we used an approach that corrects measurements for the nonstimulated control sample response (RPMI) and allows the calculation of confidence intervals and *P* values of hypothesis tests (13, 34). Only antigen response values significantly higher than the corresponding RPMI are represented, and the background for the different cytokines in the unstimulated controls never exceeded 0.05%. Analysis and presentation of distributions was performed using SPICE, version 5.1, downloaded from <http://exon.niaid.nih.gov> (35). Comparison of distributions was performed using a Student's *t* test and a partial permutation test as previously described (35). All values used for analyzing the proportionate representation of responses are background subtracted.

## RESULTS

**Generation and *in vitro* characterization of NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses.** (i) **Purity, virus growth, and plaque size phenotype.** NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses were generated as detailed in Materials and Methods, using NYVAC-WT as the parental virus. The correct insertion of HIV-1 antigens into the viral TK locus was confirmed by PCR using primers annealing in TK flanking sequences. As shown in Fig. 1A, gp140(ZM96) or Gag(ZM96)-Pol-Nef(CN54) genes were successfully inserted, and no wild-type contamination was present in NYVAC-gp140 or NYVAC-Gag-Pol-Nef preparations.

To determine if the insertion of HIV-1 genes affects virus replication, we compared the growth kinetics of NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses to that of their parental virus, NYVAC-WT, in CEF cells. Figure 1B (left) shows that the growth kinetics were similar between parental and recombinant viruses, indicating that the expression of HIV-1 antigens does not affect virus growth. As expected, in human HeLa cells there was a minimal increase in virus titer with time with NYVAC-WT or NYVAC recombinant viruses compared with replication-competent WR or Cop strains (Fig. 1B, right). The inability of NYVAC to replicate in

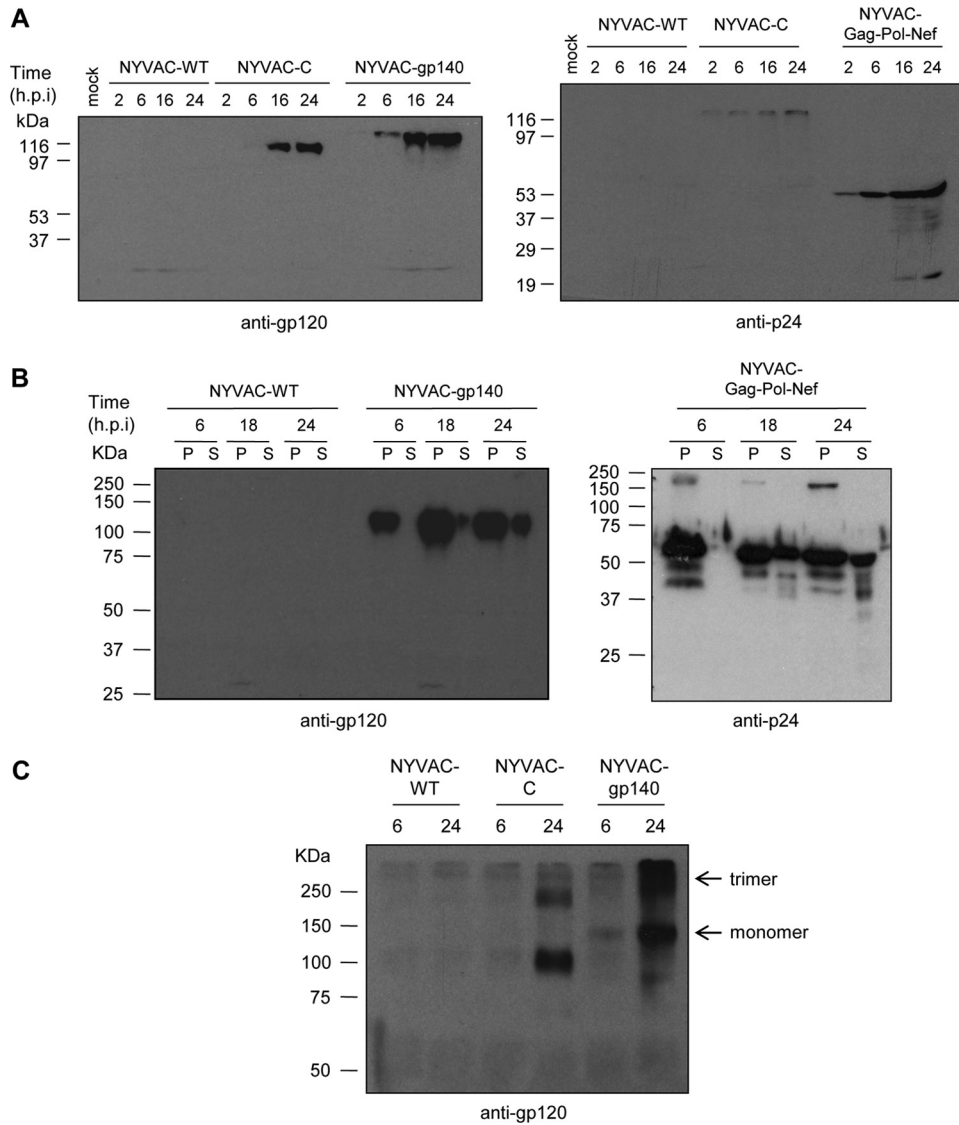
human cells is due to the lack of the host range *C7L* gene, as previously reported (4, 36). We also performed a standard plaque assay to determine the plaque size produced by both recombinant viruses compared with the parental NYVAC-WT. Figure 1C shows that NYVAC-Gag-Pol-Nef produces a plaque size phenotype similar to that produced by parental virus. However, NYVAC-gp140 plaques did not reach the size of the plaques formed by NYVAC-WT, indicating some negative effect on virion release triggered by the expression of gp140(ZM96) HIV-1 antigen.

(ii) **Time course expression analysis of HIV-1 antigens and genetic stability.** Analysis by Western blotting confirmed that the expected 140-kDa product (gp140) and the 55-kDa product obtained by the processing of the Gag(ZM96)-Pol-Nef(CN54) polyprotein are correctly expressed with time by cells infected with NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses, respectively (Fig. 2A). Both HIV-1 antigens also were detected by Western blotting in the supernatant of infected cells at 18 h postinfection (Fig. 2B). Moreover, under nonreducing conditions, gp140 appears in the supernatant of infected cells with a size compatible with the trimeric form of the Env protein (Fig. 2C). Gel filtration analysis of gp140 released from transiently transfected HEK293F cells also shows that gp140 forms trimers (data not shown). Additionally, analysis by immunostaining showed that all virus plaques have immunoreactivity to both anti-WR and anti-gp120 or anti-gag p24 antibodies (data not shown), demonstrating the stability of the HIV-1 antigens expressed by both recombinant viruses. This was further confirmed by serial passage of the NYVAC recombinant viruses in CEF cells and at passage 9, isolating 30 individual plaques and confirming by Western blotting that all plaques correctly expressed the entire gp140 and Gag antigens and by bulk DNA sequencing of each plaque that no mutations were introduced in the HIV sequences of the NYVAC vectors (data not shown).

(iii) **Intracellular localization of gp140 and Gag-Pol-Nef antigens by confocal microscopy and of Gag-induced VLP formation by electron microscopy.** The expression and intracellular localization of gp140 and Gag-Pol-Nef antigens expressed by NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses was defined by immunofluorescence analysis in BSC-40 cells using specific antibodies to HIV-1 proteins as well as specific markers for the endoplasmic reticulum (ER) (anti-PDI), Golgi membrane (WGA), and actin (phalloidin). As observed in Fig. 3A, at 6 and 24 h postinfection, gp140 protein (in green) exhibits a cytoplasmic localization within globular and dense cytoplasmic structures. These structures colocalized with ER-derived membranes (PDI; in red), as an intense change in color (yellow) with the time of infection is observed. This change in color was not observed when the Golgi membrane was stained (WGA; in red). In the case of Gag-Pol-Nef, these antigens display a more diffuse cytoplasmic distribution at different times, with some colocalization with the ER marker but not with the Golgi membrane (Fig. 3B).

Since the HIV-1 Gag proteins initially are synthesized as a polyprotein precursor which is sufficient to produce noninfectious VLPs in the absence of other viral proteins or packageable viral RNA (37), we next defined the presence of these VLPs in replication-permissive BSC-40 cells infected with NYVAC-Gag-Pol-Nef by transmission electron microscopy analysis. Ultrathin sections of infected BSC-40 cells were visualized by EM at low and high magnifications (Fig. 4 and 5). As shown in Fig. 4, at 6 and 18 h postinfection and at the single-cell level, in NYVAC-Gag-Pol-Nef-infected cells we observed an accumulation of particles lining the



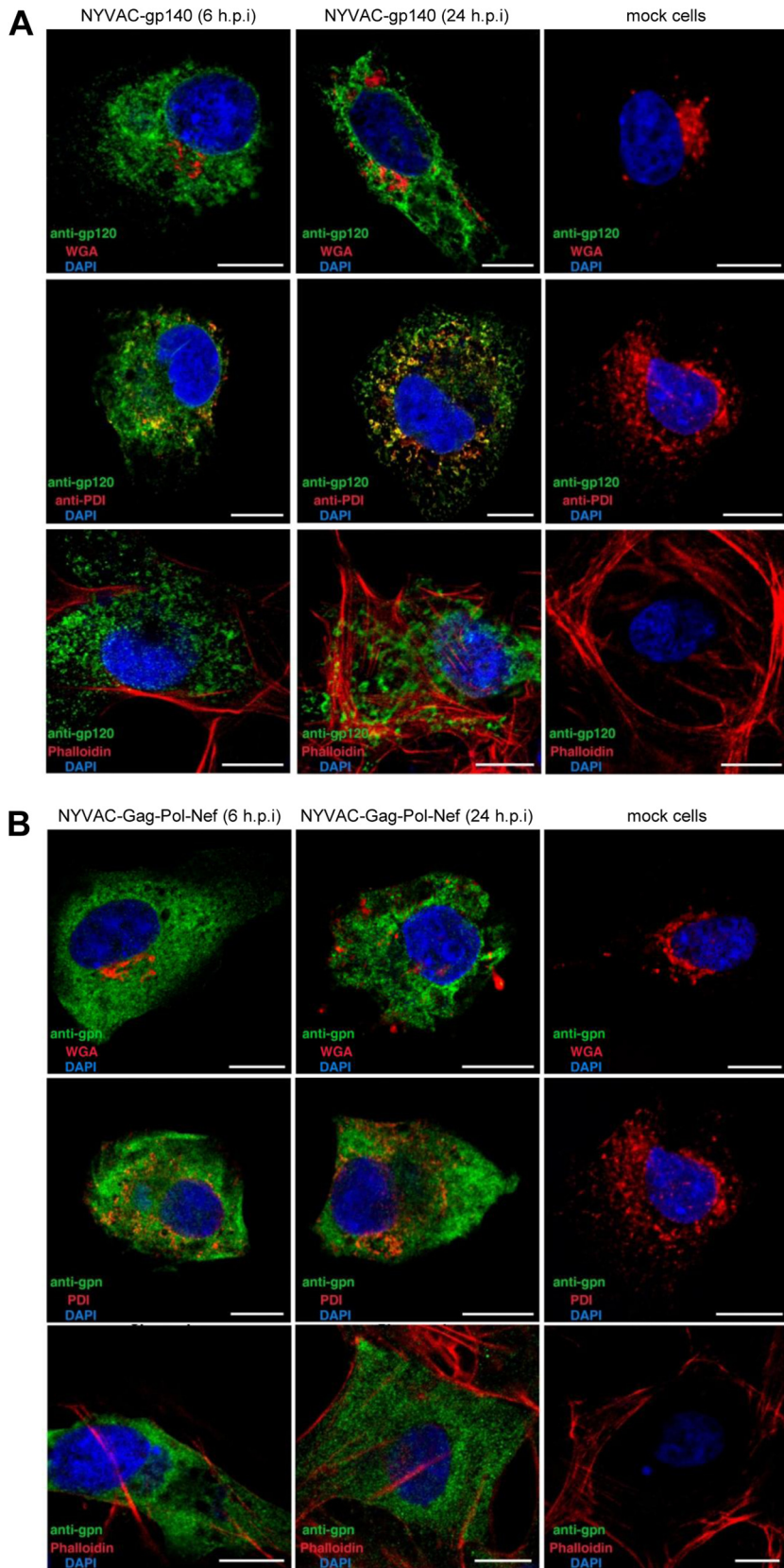


**FIG 2** Expression of gp140 and Gag-Pol-Nef antigens by Western blot analysis. (A) Time course expression of HIV-1 antigens by Western blotting. Monolayers of BSC-40 cells were mock infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-gp140, or NYVAC-Gag-Pol-Nef. At different times postinfection (2, 6, 16, and 24 h), infected cells were collected, and cells extracts fractionated by 8% SDS-PAGE and analyzed by Western blotting using the polyclonal anti-gp120 antibody or the polyclonal anti-gag p24 serum to evaluate the expression of gp140 or Gag-Pol-Nef proteins, respectively. (B) Detection of HIV-1 proteins gp140 and Gag-Pol-Nef in the supernatant of infected cells by Western blotting. Monolayers of BSC-40 cells were infected at 5 PFU/cell with NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef. At different times postinfection (6, 18, and 24 h), cells were collected and supernatants (S) and cellular pellets (P) were processed as described in Materials and Methods, fractionated by 8% SDS-PAGE, and analyzed by Western blotting using the rabbit polyclonal anti-gp120 antibody or the rabbit polyclonal anti-gag p24 serum to evaluate the expression of gp140 or Gag-Pol-Nef protein, respectively. (C) Detection of the trimeric form of gp140 in the supernatant of infected cells by Western blotting. Monolayers of BSC-40 cells were infected at 2 PFU/cell with NYVAC-WT, NYVAC-C, or NYVAC-gp140. At different times postinfection (6 and 24 h), cells were collected and supernatants processed under nonreducing conditions as described in Materials and Methods, fractionated by 8% SDS-PAGE, and analyzed by Western blotting using the rabbit polyclonal anti-gp120 antibody.

outside of the cell membrane that increases in abundance with time of infection (Fig. 4A and B). At higher magnification, dense patches lining the inner face of the plasma membrane due to Gag assembly are formed (Fig. 4C to F). Such an assembled Gag protein complex induces membrane curvature leading to the formation of a bud (Fig. 4G to H). Budding is completed as the particle pinches off from the plasma membrane (Fig. 4I to K). To confirm whether the formation of VLPs compromised the different stages in virus formation, we next examined the morphogenesis of NYVAC-Gag-Pol-Nef recombinant virus. As shown in Fig. 5A to

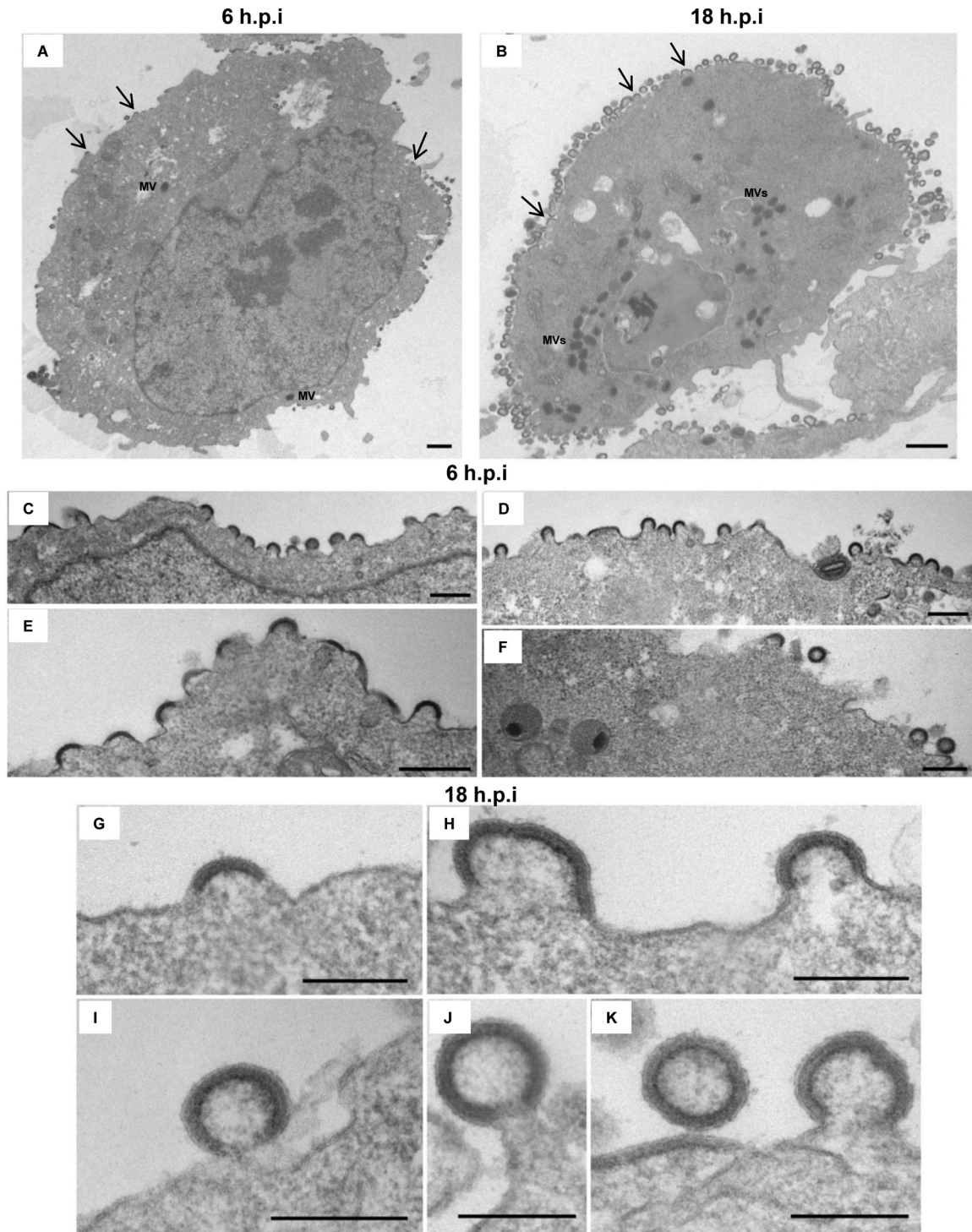
D, numerous extracellular VLPs and intracellular mature viruses (MVs) are present close to the cell surface. Virions at the cell surface can be observed in close contact with VLPs. There are also cytoplasmic immature virus forms (IVs) together with ring-shaped structures (Fig. 5E to H). In summary, the EM findings described above demonstrate that Gag-induced VLP formation does not affect VACV morphogenesis, and both NYVAC virions and VLPs coexist in the infected cell.

**Innate immune response triggered by NYVAC-gp140(ZM96) and NYVAC-Gag(ZM96)-Pol-Nef(CN54) recombinant viruses.** To



**FIG 3** Expression of gp140 and Gag-Pol-Nef proteins by confocal microscopy. BSC-40 cells at a confluence of 50% were mock infected or infected with NYVAC-gp140 or NYVAC-Gag-Pol-Nef at an MOI of 0.5 PFU/cell. At 6 or 24 h postinfection, cells were fixed, labeled with the corresponding primary antibodies followed by the appropriate fluorescent secondary antibodies, and visualized by confocal microscopy. The antibodies used were anti-gp120 (A) or anti-p24 (B) to detect HIV-1 proteins, anti-WGA to detect *trans*-Golgi membranes, anti-PDI to detect the endoplasmic reticulum, and phalloidin to detect actin. DAPI was used to detect cell nuclei. Bar, 10  $\mu$ m.

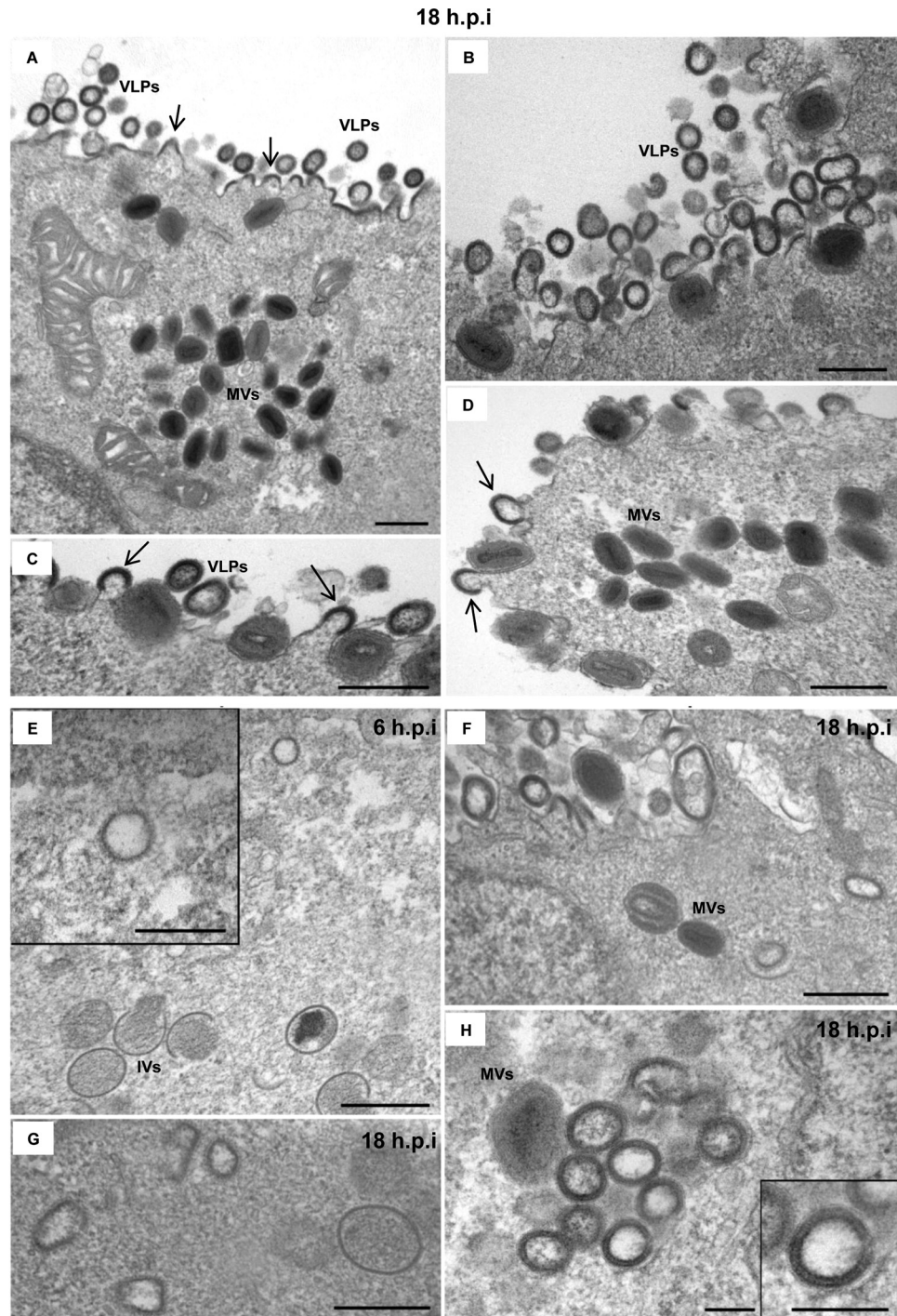




**FIG 4** Detection of VLPs generated by NYVAC-Gag-Pol-Nef-infected BSC-40 cells by electron microscopy. BSC-40 cells infected with the recombinant NYVAC-Gag-Pol-Nef at an MOI of 2.5 PFU/cell were chemically fixed at 6 or 18 hpi and then processed for conventional embedding in an epoxy resin as described in Materials and Methods. (A and B) General overview of cells infected with NYVAC-Gag-Pol-Nef at 6 or 18 hpi showing mature viruses (MVs) and singular crescent-shaped structures at the cellular membrane (arrows). Bar, 1  $\mu$ m. (C to K) Higher magnifications of NYVAC-Gag-Pol-Nef-infected cells at 6 (C to F) or 18 (G to K) hpi showing crescent-shaped budding structures at the cellular membrane and extracellular virus-like particles (VLPs). Bars: 500 nm (C to F), 250 nm (G to I), and 200 nm (J and K).

define whether HIV-1 gp140 and Gag-induced VLPs produced by the NYVAC vectors affect the innate response of immune cells, we first measured, by RT-PCR, the levels of RNA induced after virus infection (3 or 6 h; 5 PFU/cell) of human macrophages (THP-1)

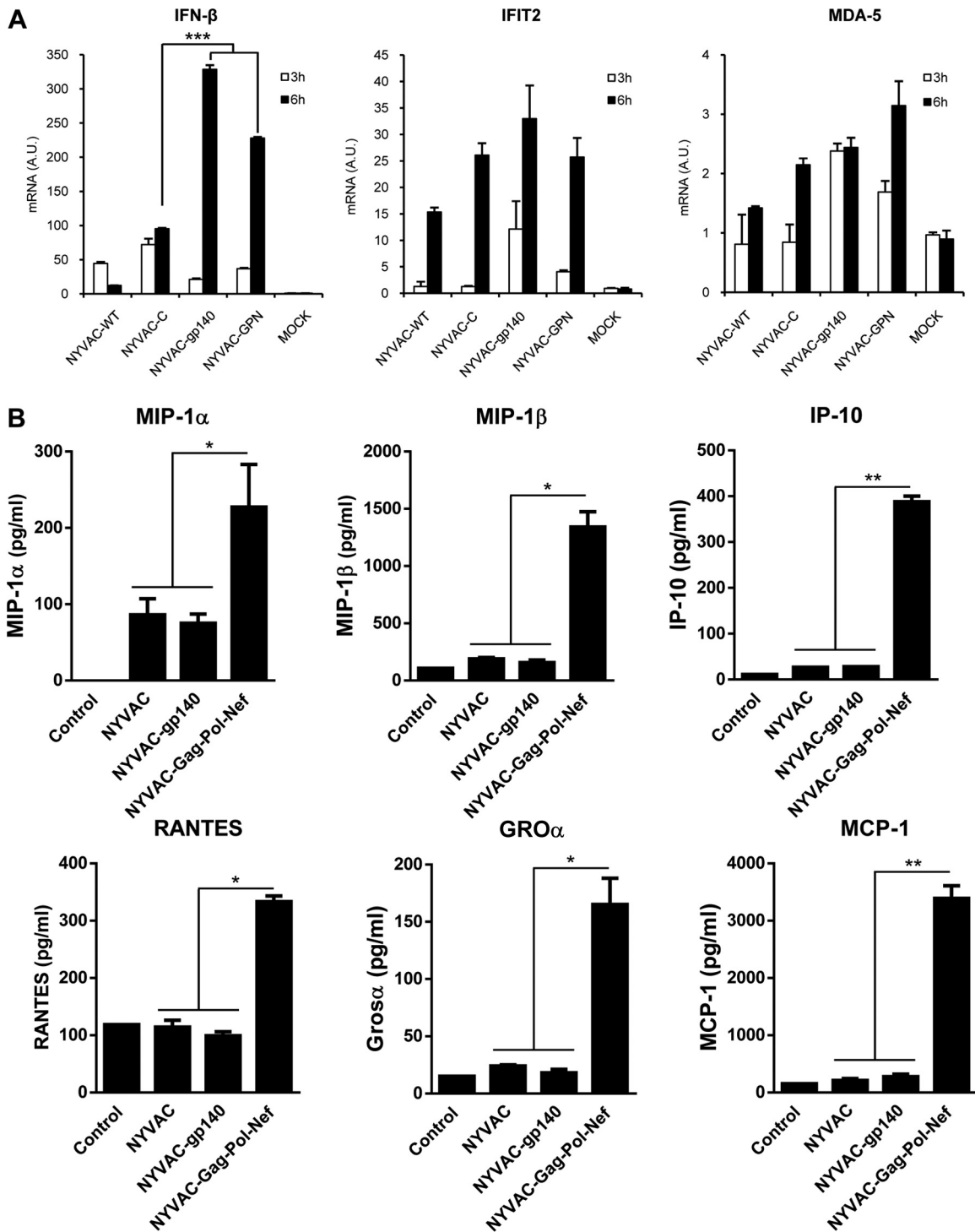
with both recombinant viruses and compared them to those of the previously described NYVAC-C vector expressing both gp120 and Gag-Pol-Nef (25). As shown in Fig. 6A, both NYVAC-gp140 and NYVAC-Gag-Pol-Nef trigger the induction of gene expression of



**FIG 5** Detection of VLPs generated by NYVAC-Gag-Pol-Nef-infected BSC-40 cells by electron microscopy (cont.). BSC-40 cells were infected with NYVAC-Gag-Pol-Nef at an MOI of 2.5 PFU/cell, chemically fixed at 6 or 18 hpi, and processed for conventional embedding in an epoxy resin. (A to D) Images of cells infected with NYVAC-Gag-Pol-Nef at 18 hpi showing intracellular mature viruses (MVs) and crescent-shaped structures (arrows) and extracellular VLPs. Bar, 500 nm. (E to H) Images of NYVAC-Gag-Pol-Nef-infected cells at 6 (E) or 18 hpi (F to H) showing intracellular immature viruses (IVs), a few MVs, and ring-shaped structures. Bars: 500 nm (E to G) and 250 nm (H).

type I interferon (IFN- $\beta$ ), of IFN-induced genes (IFIT2), and of MDA-5 to levels higher than those for NYVAC-C, providing support that the new vectors are more potent inducers of immune modulators than NYVAC-C. Since VLPs accumulate with time (Fig. 4), we next measured by Luminex the levels of several proin-

flammatory cytokines and chemokines released after 24 h of infection (1 PFU/cell) of human primary monocytes with NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef. As shown in Fig. 6B, only human monocytes infected with the vector producing VLPs induced the production of MIP-1 $\beta$ , IP-10, RANTES,



**FIG 6** Innate immune response elicited by NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses. (A) Human THP-1 macrophages were mock-infected (control) or infected with NYVAC-WT, NYVAC-C, NYVAC-gp140, or NYVAC-Gag-Pol-Nef at 5 PFU/cell, and at different times postinfection (3 or 6 h) RNA was extracted and IFN-β, IFIT2, MDA-5, and HPRT mRNA levels were analyzed by RT-PCR. Results are expressed as the ratio of the gene of interest to HPRT mRNA levels. A.U., arbitrary units. (B) Human primary monocytes from 1 healthy volunteer were mock infected (control) or infected for 24 h with NYVAC-WT, NYVAC-gp140, or NYVAC-Gag-Pol-Nef at an MOI of 1 PFU/cell, and concentrations of MIP-1α, MIP-1β, IP-10, RANTES, GROα, and MCP-1 were measured in cell culture supernatants by the Luminex technology. Data are means ± standard deviations (SD) of duplicates samples from one experiment and are representative of two donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ .



GRO $\alpha$ , and MCP-1. NYVAC-Gag-Pol-Nef also induced a stronger production of MIP-1 $\alpha$  than NYVAC-WT and NYVAC-gp140. Thus, the expression of VLPs by NYVAC-Gag-Pol-Nef recombinant virus triggers a stronger innate immune response than either NYVAC-WT or NYVAC-gp140 at late times.

**NYVAC-gp140 and NYVAC-Gag-Pol-Nef elicit an attenuated profile in immunocompromised and newborn mice.** To assess the attenuation profile of NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses, we first immunocompromised adult mice by cyclophosphamide treatment, since we have previously shown that this drug is effective for the study of viral pathogenesis (28). Groups of six BALB/c mice that had been cyclophosphamide treated or left untreated were i.c. mock infected with PBS or infected with  $10^5$ ,  $10^6$ , or  $10^7$  PFU/animal of the Copenhagen strain or with  $10^6$ ,  $10^7$ , or  $10^8$  PFU/animal of NYVAC recombinant viruses. Loss of weight and other signs of illness were monitored daily after infection with the different viruses for up to 30 days postinoculation, and animals that lost 25% or more of their initial weight were euthanized. A scheme of the immunization schedule is represented in Fig. 7A, and pathogenicity results are shown in Fig. 7B. Drug-treated and untreated animals infected with  $10^6$  or  $10^7$  PFU of the Copenhagen strain exhibited severe loss of weight and developed signs of illness, such as rough coat hair, loss of mobility, and hunched posture (data not shown), and had to be sacrificed at day 3 postinfection. However, at a dose of  $10^5$  PFU of the Copenhagen strain, all drug-treated animals died by 5 days postinfection, while 50% of the untreated animals infected with the same dose survived the course of the study (Fig. 7B, upper row). In contrast, all drug-treated animals infected with NYVAC-gp140 or NYVAC-Gag-Pol-Nef survived at doses of  $10^6$  and  $10^7$  PFU, showing mild signs of illness compared to the Copenhagen virus (data not shown), since animals infected with  $10^8$  PFU died by 9 (NYVAC-Gag-Pol-Nef; Fig. 7B, middle row) or 11 (NYVAC-gp140; Fig. 7B, lower row) days postinfection. The progression of the weight of the surviving animals in each group also is represented (Fig. 7B, left). Therefore, the results described above demonstrate that NYVAC-gp140 and NYVAC-Gag-Pol-Nef are highly attenuated in adult mice even when using the intracranial inoculation route in the context of immunocompromised animals.

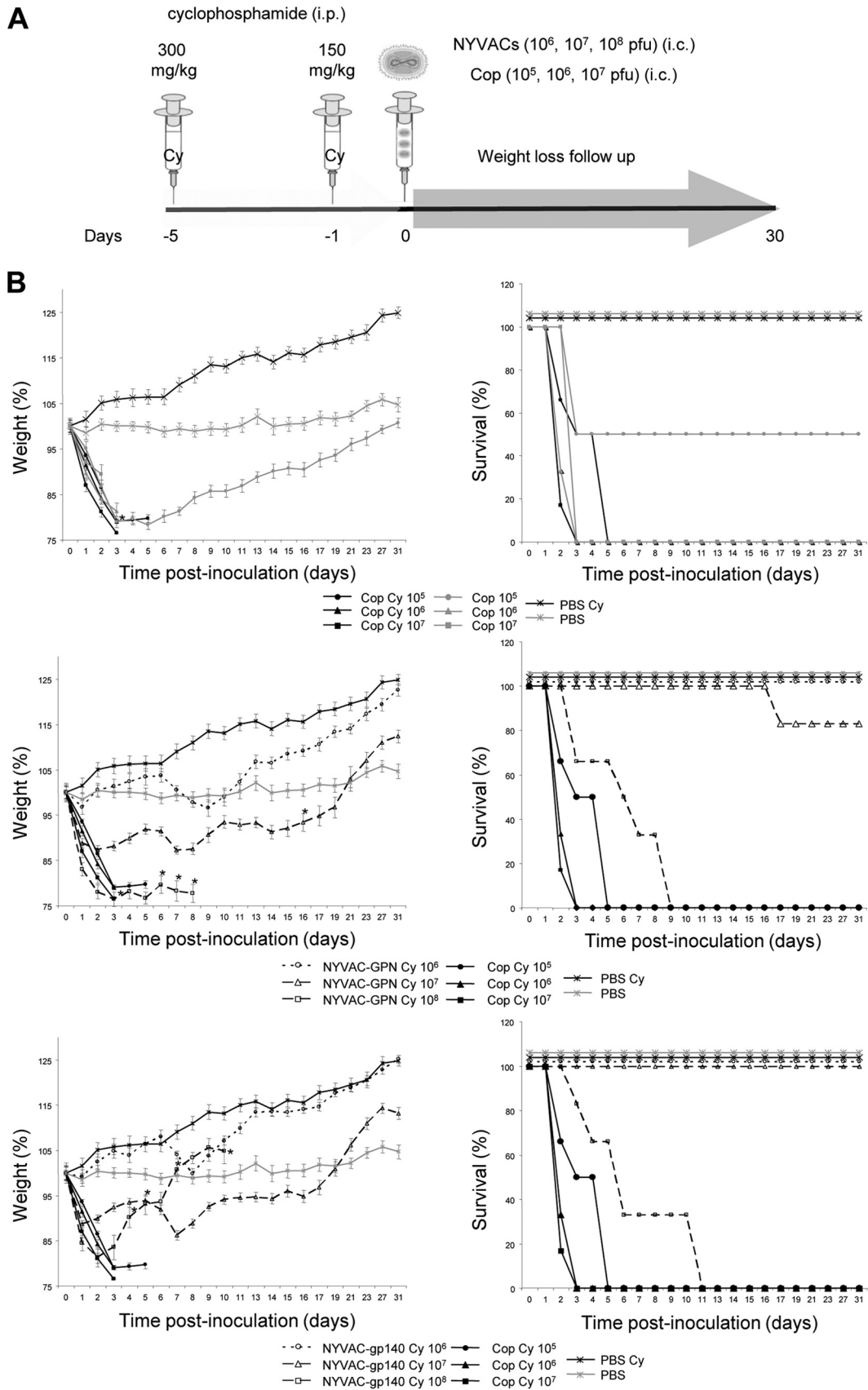
To further document the attenuation profile of the NYVAC vectors, we next performed i.c. inoculations in newborn CD1 mice (10 per group) with different doses of vectors and monitored signs of morbidity/mortality twice daily for 14 days. While Copenhagen strain VC-2, from which NYVAC was derived after the precise deletion of 18 open reading frames, has a 50% lethal dose (LD<sub>50</sub>) close to 1 PFU, the LD<sub>50</sub> for animals inoculated with the NYVAC vectors required doses higher than  $10^6$  PFU. In these experiments we compared the novel NYVAC vectors, NYVAC-gp140 and NYVAC-Gag-Pol-Nef, to the old vector NYVAC-C, expressing gp120 and Gag-Pol-Nef in the same viral genome, that has shown good safety profiles in phase I clinical trials (20, 21). The results of Fig. 7 and 8 demonstrate the high-attenuation profile of the different NYVAC vectors expressing distinct HIV antigens in two different mouse models.

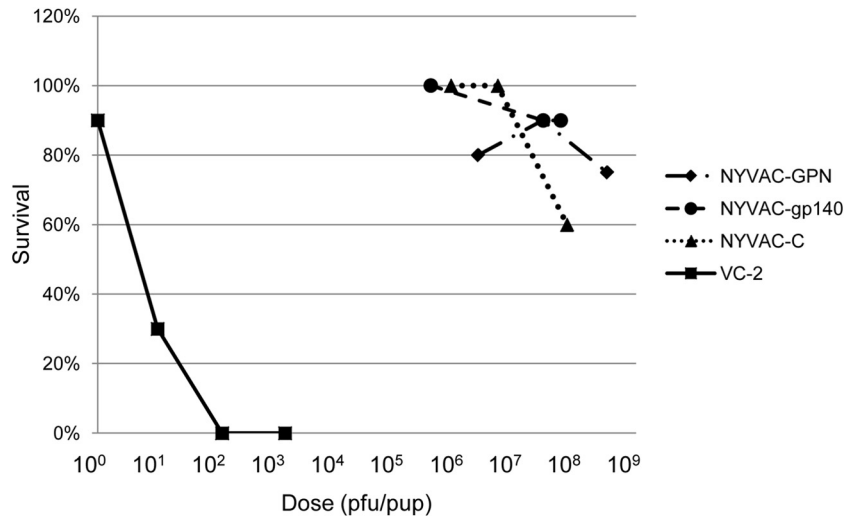
**Polyfunctional gp140 or Gag-specific T cell adaptive immune responses induced by NYVAC-gp140 or NYVAC-Gag-Pol-Nef in BALB/c mice in homologous prime/boost combination.** To assay *in vivo* the effect of NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses on immunogenicity triggered by HIV-1 gp140 or Gag-Pol-Nef antigens, we analyzed the HIV-1-specific T

cell adaptive immune responses elicited in mice using a poxvirus prime/boost immunization approach. BALB/c mice, 4 in each group, were immunized with a total of  $2 \times 10^7$  PFU/mouse as described in Materials and Methods, and HIV-1-specific CD4 and CD8 T cell adaptive immune responses were measured 10 days after the last immunization by ICS assay (Fig. 9A). We used four groups of animals: group 1, NYVAC-WT/NYVAC-WT; group 2, NYVAC-gp140/NYVAC-gp140; group 3, NYVAC-GPN/NYVAC-GPN; and group 4, NYVAC-MIX/NYVAC-MIX (mixing equal amounts of NYVAC-gp140 and NYVAC-GPN). Splenocytes from immunized animals were stimulated *ex vivo* for 6 h with a panel of 166 peptides from HIV-1 ZM96 gp140 grouped in three different Env peptide pools, Env-1, Env-2, and Env-3, or with the peptide Gag(ZM96) or Pol-1 and stained with specific antibodies to identify T cell lineage (CD3, CD4, and CD8), degranulation (CD107a), and responding cells (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ). The percentages of T cells producing IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$  established the overall CD4<sup>+</sup> T cell responses, whereas the percentages of T cells producing CD107a, IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$  determined the overall CD8<sup>+</sup> T cell responses. Results are presented for groups 2 to 4, as the nonspecific responses in the parental NYVAC-WT group were subtracted in all groups. The main gp140-specific T cell immune response detected was due to the CD4 compartment and was directed against the Env-1 peptide pool (Fig. 9). As shown in Fig. 9B, in both immunization groups, NYVAC-gp140/NYVAC-gp140 and NYVAC-MIX/NYVAC-MIX, the magnitudes of the Env-1-specific CD4 T cell responses were significantly higher than that obtained in the group NYVAC-GPN/NYVAC-GPN ( $P < 0.001$ ). Furthermore, the magnitude of the Env-1-specific CD4 T cell responses in the group immunized with NYVAC-gp140 was significantly higher than that obtained in the group immunized with NYVAC-MIX ( $P < 0.005$ ), which is consistent with the lower (by half) virus dose of NYVAC-gp140 used in the NYVAC-MIX immunization group. Representative functional profiles of Env-1-specific CD4 T cell responses are shown in Fig. 9C.

The quality of a T cell response can be characterized by the profile of cytokine production and by the cytotoxic potential. Based on the analysis of IFN- $\gamma$ , IL-2, and TNF- $\alpha$  secretion, as well as the study of CD107a expression on the surface of activated T cells as an indirect marker of cytotoxicity, 7 different HIV-1-specific CD4 T cell populations were induced after immunization with NYVAC-gp140 or NYVAC-MIX. Env-1-specific CD4 T cell responses were highly polyfunctional in both NYVAC-gp140 and NYVAC-MIX immunization groups, with more than 75% of CD4<sup>+</sup> T cells exhibiting two or three functions (Fig. 9D). CD4 T cells producing IFN- $\gamma$ , IL-2, and TNF- $\alpha$  or IL-2 and TNF- $\alpha$  were the most representative populations induced in both immunization groups, with the NYVAC-gp140 immunization group inducing significantly higher magnitudes of these populations than NYVAC-MIX. Moreover, in the group immunized with NYVAC-gp140, CD4 T cells producing IFN- $\gamma$  and TNF- $\alpha$  or only IL-2 or TNF- $\alpha$  also were detected (Fig. 9D).

Regarding Gag-specific T cell responses (Fig. 10), in both immunization groups NYVAC-GPN/NYVAC-GPN and NYVAC-MIX/NYVAC-MIX, the Gag-Pol-Nef-specific T cell response detected was due to the CD8 compartment and was directed against Gag(ZM96) peptide (Fig. 10A). The magnitudes of the Gag-specific CD8 T cell responses were significantly higher than that obtained in the group NYVAC-gp140/NYVAC-gp140 ( $P < 0.001$ ), but no statistical differences were observed between the magni-





**FIG 8** Neurovirulence in newborn mice by intracranial inoculation. Newborn CD1 mice (minimum of 10 per group) were inoculated i.c. with the indicated viruses at the indicated doses (PFU). Mice were monitored twice daily for 14 days for signs of morbidity/mortality. Results are adjusted for titration values determined after inoculation.

tudes of the Gag-specific CD8 T cell responses in the groups immunized with NYVAC-GPN or NYVAC-MIX (Fig. 10A). Representative functional profiles of Gag-specific CD8 T cell responses are shown in Fig. 10B.

Gag-specific CD8 T cell responses were highly polyfunctional in both NYVAC-GPN and NYVAC-MIX immunization groups, with more than 75% (NYVAC-GPN) or 50% (NYVAC-MIX) of CD8<sup>+</sup> T cells exhibiting two, three, or four functions (Fig. 10C). Fifteen different HIV-1-specific CD8 T cell populations were induced after immunization with NYVAC-GPN or NYVAC-MIX. CD8 T cells producing CD107a, IFN- $\gamma$ , and TNF- $\alpha$  or only CD107a were the most representative populations induced in both immunization groups (Fig. 10C).

Overall, these results indicate that both NYVAC-gp140 and NYVAC-GPN recombinant viruses induce highly polyfunctional HIV-1-specific T cell immune responses.

**NYVAC-gp140 and NYVAC-Gag-Pol-Nef induce a humoral response against HIV-1 Env and Gag antigens.** Since cells infected with NYVAC-gp140 release trimeric gp140 protein and NYVAC-Gag-Pol-Nef produces Gag-induced VLPs, we also evaluated the effect of both recombinant viruses on antibody responses. Thus, we quantified by ELISA the reactivity of serum from individual animals against the purified trimeric gp140 or p17/p24 antigens. Specific IgG titers are shown in Fig. 11. Clearly, all mice that received NYVAC-gp140 produced high titers of antibodies against gp140 (Fig. 11A). Reactivity against p17/p24 also was observed in all mice immunized with NYVAC-Gag-Pol-Nef (Fig. 11B), although titers were lower than those for gp140. These results demonstrate that the bivalent NYVAC vectors are good inducers of humoral responses in mice.

## DISCUSSION

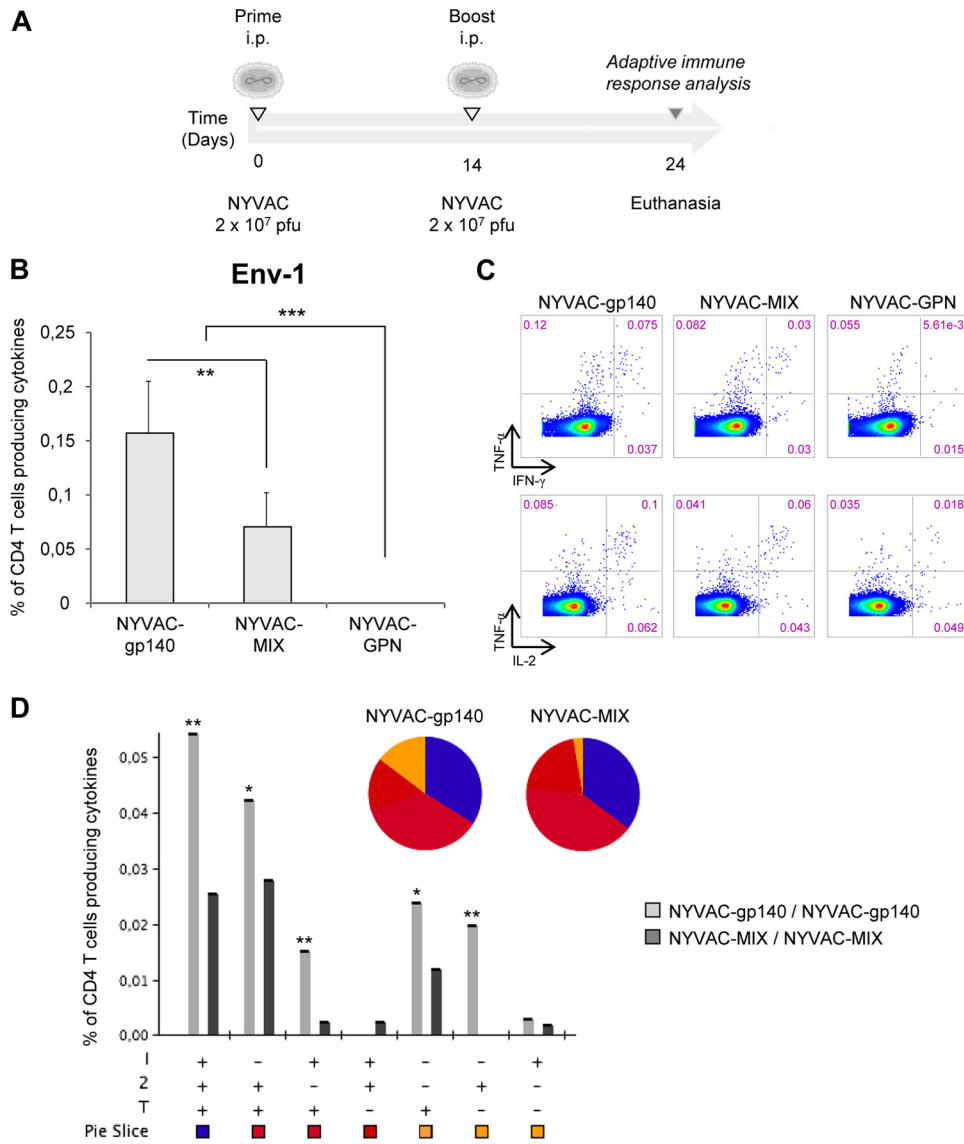
Development of attenuated VACV-based vectors with enhanced immunogenicity as vaccine candidates against HIV-1 antigens in humans is a challenging goal in the poxvirus and HIV/AIDS fields. The restricted immunogenicity triggered in different clinical trials by attenuated poxvirus vectors expressing HIV-1 antigens (3) and the modest efficacy (31.2%) against HIV infection obtained in the RV144 phase III clinical trial (1) pointed to the need for generating poxvirus vectors with improved immunogenicity and to learn more about virus-host cell interactions. In this context, different strategies have been followed (3, 8–19).

While a variety of attenuated poxvirus vectors expressing HIV antigens have been developed (17, 18), here we describe a different strategy to improve the immunogenicity against HIV-1 antigens. This strategy is based on the optimization of the HIV-1 antigens inserted in the poxvirus vector. With this aim, we have selected two different HIV-1 antigens: a trimeric soluble gp140 protein from HIV-1 clade C ZM96 and a Gag(ZM96)-Pol-Nef(CN54) polyprotein (also from HIV-1 clade C), which was specifically designed to be processed to give a 55-kDa Gag protein able to induce the formation of VLPs. Both HIV-1 antigens have been inserted separately into the TK locus of the NYVAC genome, resulting in the generation of NYVAC-gp140 and NYVAC-Gag-Pol-Nef recombinant viruses, respectively.

In the present study, we have shown that the insertion of the HIV-1 genes encoding gp140 or Gag-Pol-Nef antigens in the NYVAC genome had no effect on the replication capacity of the virus in CEF cells, which is relevant for the manufacture of viral stocks, although in the case of NYVAC-gp140 the insertion of

**FIG 7** Neurovirulence in cyclophosphamide-treated immunocompromised mice by intracranial inoculation. (A) Immunization schedule. BALB/c mice (6 to 8 weeks old; 6 per group) received 300 mg/kg cyclophosphamide (Cy) by the i.p. route 5 days before the immunization and a second dose of 150 mg/kg 24 h before the inoculation of the different viruses. Cyclophosphamide-treated mice received 10<sup>6</sup>, 10<sup>7</sup>, or 10<sup>8</sup> PFU of NYVAC-gp140 or NYVAC-Gag-Pol-Nef or 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> PFU of Cop virus by the intracranial (i.c.) route as described in Materials and Methods. (B) Monitoring of weight (left column) and mortality (right column) with time. Symbols are given for each virus. Identical data from control groups (Cop Cy, PBS Cy, and PBS) are depicted in the different graphics. Asterisks indicate days of sacrifice.





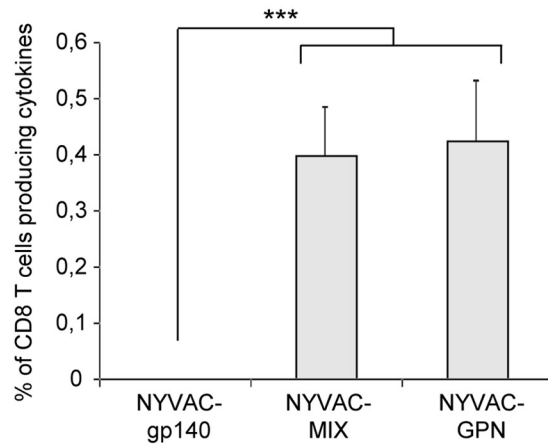
**FIG 9** Adaptive HIV-1-specific T cell immune response elicited by NYVAC-gp140 recombinant virus in the spleen of BALB/c mice in a homologous prime/boost immunization protocol. (A) Immunization schedule. BALB/c mice (6 to 8 weeks old;  $n = 4$  per group) were immunized with  $2 \times 10^7$  PFU of NYVAC-WT, NYVAC-gp140, NYVAC-Gag-Pol-Nef, or NYVAC-MIX ( $1 \times 10^7$  PFU of NYVAC-gp140 plus  $1 \times 10^7$  PFU of NYVAC-Gag-Pol-Nef) by the intraperitoneal (i.p.) route. Two weeks later, animals were immunized as described for the priming, and 10 days after the last immunization, mice were sacrificed and spleens processed for intracellular cytokine staining (ICS). Two independent experiments have been performed for the different groups. (B) Magnitude of the vaccine-specific CD4 T cell response. The HIV-specific CD4 T cells were measured 10 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals with the different gp140(ZM96) peptide pools. The total value in each group represents the sum of the percentages of CD4<sup>+</sup> T cells secreting IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$  against the Env-1 peptide pool. All data are background subtracted. \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.001$ . (C) Flow cytometry profiles of vaccine-induced CD4 T cell responses against the Env-1 pool. (D) Functional profile of the adaptive HIV-specific CD4 T cell response in the different immunization groups against the Env-1 peptide pool. The possible combinations of the responses are shown on the x axis, whereas the percentages of the functionally distinct cell populations within the total CD4 T cell population are shown on the y axis. Responses are grouped and color coded on the basis of the number of functions. The nonspecific responses obtained in the control NYVAC group were subtracted in all populations. I, IFN- $\gamma$ ; 2, IL-2; T, TNF- $\alpha$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .  $P$  values indicate significantly higher responses than the NYVAC-MIX immunization group.

gp140 affects the plaque size phenotype, probably due to some negative effect exerted by gp140. Moreover, the HIV-1 antigens inserted in the NYVAC genome are stable, as no modifications on HIV-1 antigen expression were observed after 9 consecutive passages of the vectors in CEF cells. This was further confirmed after bulk sequencing of the HIV inserts from 30 individual virus plaques isolated after passage 9. Both gp140 and Gag-Pol-Nef antigens are correctly expressed and released with time from the cell,

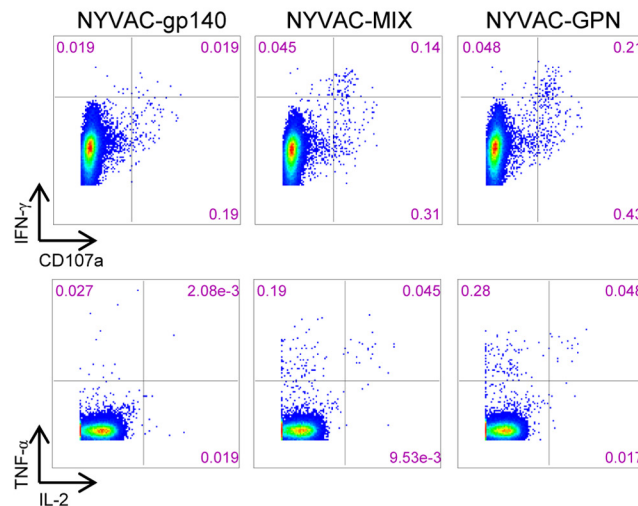
as demonstrated by the detection of these HIV-1 antigens in the supernatant of infected cells, and gp140 has been shown to produce trimeric soluble forms under nonreducing conditions. As observed by electron microscopy, Gag-induced VLPs are produced abundantly at the cell surface of virus-infected cells, and complete particles are released with time from the cell. Interestingly, the VLP accumulation can coexist with the production of infectious forms of NYVAC, as both VLPs and NYVAC virions are

**A**

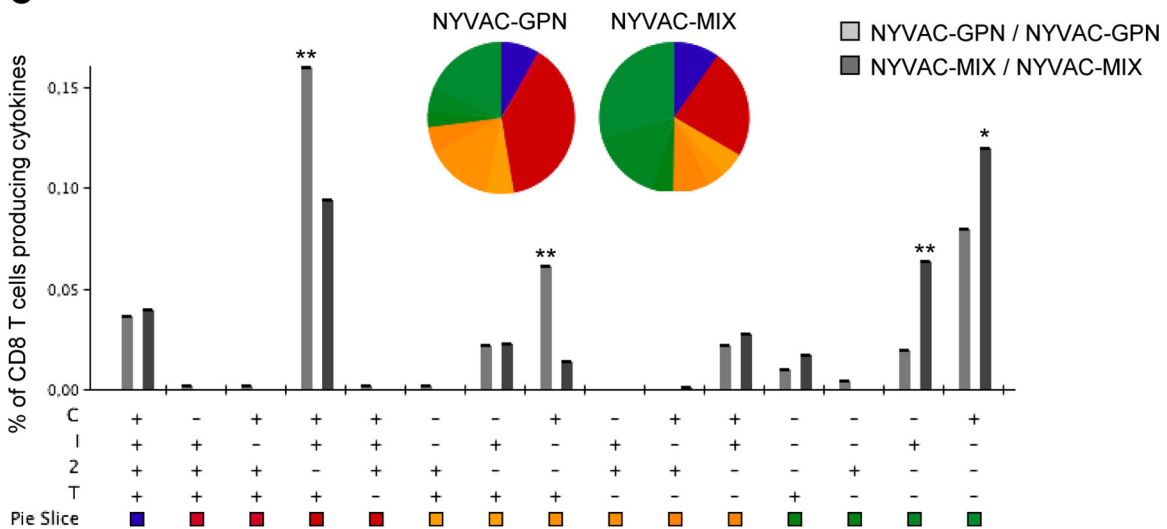
**Gag (ZM96)**



**B**



**C**



**FIG 10** Adaptive HIV-1-specific T cell immune response elicited by NYVAC-Gag-Pol-Nef recombinant virus in the spleen of BALB/c mice in a homologous prime/boost immunization protocol. (A) Magnitude of the vaccine-specific CD8 T cell response. The HIV-specific CD8 T cells were measured 10 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals with Gag(ZM96) peptide. The total value in each group represents the sum of the percentages of CD8<sup>+</sup> T cells secreting IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and/or CD107a against Gag(ZM96) peptide. All data are background subtracted. \*\*\*,  $P < 0.001$ . (B) Flow cytometry profiles of vaccine-induced CD8 T cell responses against Gag(ZM96) peptide. (C) Functional profile of the adaptive HIV-specific CD8 T cell response in the different immunization groups against Gag(ZM96) peptide. The possible combinations of the responses are shown on the x axis, whereas the percentages of the functionally distinct cell populations within the total CD8 T cell population are shown on the y axis. Responses are grouped and color-coded on the basis of the number of functions. The nonspecific responses obtained in the control NYVAC group were subtracted in all populations. C, CD107a; I, IFN- $\gamma$ ; 2, IL-2; T, TNF- $\alpha$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ .

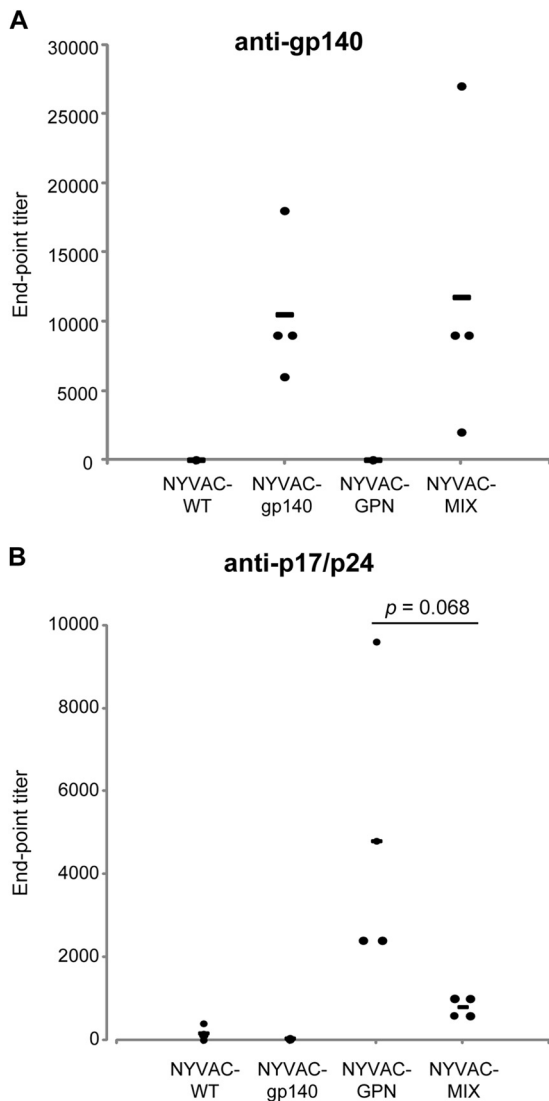


FIG 11 Adaptive humoral immune response elicited by NYVAC-gp140 and NYVAC-Gag-Pol-Nef. Levels of gp140 (A) or p17/p24 (B)-specific IgG binding antibodies were measured in serum from naive and immunized individual mice 10 days after the last immunization by ELISA. ●, antibody titers for each individual mouse; —, mean antibody titers for each group.

found together within and at the cell surface. The coexistence of NYVAC virions with Gag VLPs is relevant for the use of replication-competent viruses in human cells as candidate vaccines.

While the parental NYVAC vector has been shown to trigger a more proinflammatory phenotype in immune cells than other vectors (38, 39), the novel NYVAC vectors described here induced more potent innate responses than the old NYVAC-C vector. As shown in Fig. 6A, both NYVAC-gp140 and NYVAC-Gag-Pol-Nef trigger the induction of the gene expression of IFN- $\beta$ , IFIT2, and MDA-5 to levels higher than those for NYVAC-C. At late times postinfection, there is higher induction of cytokines/chemokines for NYVAC producing VLPs than for NYVAC or NYVAC-gp140, probably due to the interaction of VLPs with cells that, in turn, enhanced the induction of specific immune modulators. Additionally, both recombinant viruses have been demonstrated to be highly attenuated in immunocompromised adult animals as well

as in newborn mice inoculated by the i.c. route, attesting to the high safety profiles.

We next wanted to determine whether all of the above-described observations (features of HIV antigens released from cells, structural organization, proinflammatory environment triggered by the activation of specific signaling pathways, and the attenuated phenotype exerted in mice) contribute to the nature of the T and B cell immune responses elicited by the NYVAC recombinant vectors. Although it would have been of interest to compare the immunogenicity of the old NYVAC-C vector used in clinical trials (20–22) with the one elicited by the new NYVAC vectors, a direct head-to-head immunological comparison cannot be done properly, as the HIV inserts are different between the NYVAC vectors, where subtype CN54 is present in the old NYVAC-C vector and ZM96 is present in Env and Gag of the new NYVAC vectors; this was confirmed experimentally after direct comparison of NYVAC-C to the new NYVACs in immunized mice (data not shown).

In the BALB/c mouse model, immunization with NYVAC-gp140 induced polyfunctional Env-specific CD4 T cell responses, while animals immunized with NYVAC-Gag-Pol-Nef triggered a preferential Gag-specific CD8 T cell immune response. We observed that mixing the two vectors and inoculating them together in the animal, compared with single-vector immunization, elicited significant immune responses to both Env and Gag, but the magnitude was dose related for gp140 and less influenced for Gag expression. The comparison of the T cell populations activated, in terms of magnitude and polyfunctional profile of Env- and Gag-specific T cell immune responses, revealed that both gp140 and Gag-Pol-Nef induced a differential T cell response, being highly polyfunctional CD4 T cells in the case of gp140 and less polyfunctional but higher in magnitude and of the CD8 phenotype in the case of Gag-Pol-Nef. These observations suggest that the expression of HIV-1 antigens from different vectors is a valid strategy to avoid the immunodominance of Env, to obtain a more balanced Env/Gag-Pol-Nef T cell response, and to trigger better humoral (through gp140-specific CD4 T cell responses) and cellular (through Gag-specific CD8 T cell responses) immune responses. This could be explained by the specific nature of the HIV-1 inserts: (i) Env is targeted via the signal peptide directly to the ER and subsequently secreted without allowing access to the proteasomal compartment, whereas (ii) Gag-Pol-Nef is translated in the cytoplasm, where at least part of the protein can access the proteasomal pathway. In this context, it has been demonstrated previously that Gag VLPs *per se* can enter the major histocompatibility complex class I processing and presentation pathways (40). Therefore, the observations reported in the present work are particularly relevant in view of the results obtained in phase I clinical trials with a NYVAC vector coexpressing both gp120 and Gag-Pol-Nef (NYVAC-C), where a biased CD4 T cell response for Env was observed (20, 21). On the other hand, the specific design of both antigens, gp140 as a soluble trimeric complex and Gag-Pol-Nef as a polyprotein able to be processed and to produce Gag-induced VLPs, also has influenced in a positive manner the humoral response elicited by both recombinant viruses. In fact, high-titer antibodies against HIV-1 gp140 antigen were obtained in animals immunized with NYVAC-gp140.

In summary, in the present study we have engineered two novel NYVAC-based vectors as HIV vaccine candidates, established the stability of the HIV inserts by bulk sequencing following multiple passages of the NYVAC vectors, and defined kinetic stud-



ies and extracellular release of HIV antigens. We also determined the localization and morphology of intra- and extracellular VLPs by electron microscopy, the lack of cellular interference between VLPs and NYVAC virions, the specificity in the induction of innate immune responses, the degree of attenuation in two animal models (immunocompromised and newborn), the magnitude of HIV-1-specific CD4 and CD8 T cell responses, and the induction of antibody responses to Env and p17/p24. Overall, this study provides deep insights into NYVAC-HIV vectors based on the virological and immunological responses induced in a mouse pre-clinical model and represents an important contribution to virus-host cell interactions of NYVAC vectors expressing distinct HIV antigens. Moreover, we have defined specific immune parameters which will help to unravel potential correlates of protection against HIV in human clinical trials with these vectors.

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