Journal of Virology

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***J. Virol.* 2012, 86(9):5026. DOI: 10.1128/JVI.06684-11.**
Published Ahead of Print 14 March 2012.

JVI Journals.ASM.org

Removal of Vaccinia Virus Genes That Block Interferon Type I and II Pathways Improves Adaptive and Memory Responses of the HIV/AIDS Vaccine Candidate NYVAC-C in Mice

Carmen Elena Gómez,[a] Beatriz Perdiguero,[a] Jose Luis Nájera,[a] Carlos Oscar S. Sorzano,[b] Victoria Jiménez,[a] Rubén González-Sanz,[a] and Mariano Esteban[a]

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Madrid, Spain,[a] and Biocomputing Unit, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Madrid, Spain[b]

Poxviruses encode multiple inhibitors of the interferon (IFN) system, acting at different levels and blocking the induction of host defense mechanisms. Two viral gene products, B19 and B8, have been shown to act as decoy receptors of type I and type II IFNs, blocking the binding of IFN to its receptor. Since IFN plays a major role in innate immune responses, in this investigation we asked to what extent the viral inhibitors of the IFN system impact the capacity of poxvirus vectors to activate immune responses. This was tested in a mouse model with single and double deletion mutants of the vaccine candidate NYVAC-C, which expresses the HIV-1 Env, Gag, Pol, and Nef antigens. When deleted individually or in double, the type I (B19) and type II (B8) IFN binding proteins were not required for virus replication in cultured cells. Studies of immune responses in mice after DNA prime/NYVAC boost revealed that deletion of *B8R* and/or *B19R* genes improved the magnitude and quality of HIV-1-specific $CD8^+$ T cell adaptive immune responses and impacted their memory phase, changing the contraction, the memory differentiation, the effect magnitude, and the functionality profile. For B cell responses, deletion of the viral gene *B8R* and/or *B19R* had no effect on antibody levels to HIV-1 Env. These findings revealed that single or double deletion of viral factors (B8 and B19) targeting the IFN pathway is a useful approach in the design of improved poxvirus-based vaccines.

The generation of vaccines that induce long-lived protective immunity against HIV-1 infection remains a major, challenging goal. However, the recent observation of approximately 31% protection against HIV-1 infection in a phase III clinical trial (RV144) in Thailand that evaluated a combination of the recombinant poxvirus vector ALVAC and the protein gp120 (44) highlighted that improved poxvirus recombinants should be considered components of an effective HIV/AIDS vaccine (18, 35).

Among the poxviruses, the highly attenuated vaccinia virus (VACV) strain NYVAC is nowadays under intense preclinical and clinical evaluation as a vaccine against emergent infectious diseases and cancer (18). The NYVAC strain was derived from a plaque clone isolate of the Copenhagen vaccinia virus strain (VACV-COP) by the precise deletion of 18 open reading frames (ORFs) implicated in pathogenesis, virulence, and host range regulatory functions (49). Despite its limited replication in human and most mammalian cell types, NYVAC provides a high level of gene expression and triggers antigen-specific immune responses when delivered to animals and humans (14, 18, 36, 38). However, the vector still contains other viral genes with immunomodulatory functions that may suppress host immunity, in particular, genes encoding proteins that antagonize the interferon (IFN) system (37).

Among the key elements of host innate immunity that prevent the pathogenesis of virus-induced diseases are the IFNs. IFNs play an important role in protection against infection by a large number of viruses, including VACV and other poxviruses (13, 33, 45, 53). To evade the antiviral effects, VACV counteracts the IFN system by viral expression of a number of different factors, including soluble IFN receptors and intracellular proteins that block the activities of key IFN-induced genes (37). IFNs fall into three classes, designated types I to III, and are classified according to the receptor complex through which they signal. Type I IFNs are represented by various IFN-α subtypes, IFN-β, IFN-ω, and IFN-τ. All these are essential for mounting a robust host response against viral infection and bind to a common heterodimeric receptor, IFN-α/βR, which is ubiquitously expressed (39). IFN-γ (type II IFN) binds to its cognate receptor on cells, IFN-γR (1). It is secreted by activated T cells and natural killer (NK) cells, rather than in direct response to viral infection, being a potent inducer of the cell-mediated (Th1) immune response. The more recently described type III IFNs are represented by various IFN-λ subtypes and bind to a unique receptor containing the interleukin-10 receptor β (IL-10Rβ) and IL-28Rα subunits (26). They are known to regulate the antiviral response and have been proposed to be the ancestral type I IFNs. Considering the host defense that is mounted by the IFN system to fight viral infections, it is not surprising that VACV uses different gene products and strategies to prevent the IFN effects.

The VACV type I vIFN-α/βR homolog (B19 in strain Copenhagen and B18 in strain WR) is a glycoprotein expressed early during infection (9). It has been detected as both a secreted protein and attached to the cell surface of infected and uninfected cells via an interaction with glycosaminoglycans, suggesting that it pro-

Received 28 October 2011 **Accepted** 22 February 2012
Published ahead of print 14 March 2012
Address correspondence to Mariano Esteban, mesteban@cnb.csic.es.
C.E.G. and B.P. contributed equally to this study.
Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.06684-11

tects infected cells from the direct action of IFN-α/β and uninfected cells from IFN-induced resistance to infection (4, 29). In contrast to the cellular receptors, the viral protein binds and inhibits type I IFNs from a broad range of species, including human, cow, rabbit, rat, and mouse, although the affinity for mouse type I IFNs is considerably lower than for the other species tested (48). Recently, it was determined that vIFN-α/βR inhibits only IFN-α and not IFN-β in murine dendritic cells (DCs) (55). On the other hand, the VACV type II vIFN-γR homolog (B8) is a glycoprotein that is secreted as a homodimer from infected cells early during infection (2). It has a remarkably broad specificity and is able to bind and inhibit IFN-γ from humans and cows, rats, rabbits, chickens, and horses but not mice (3, 31).

Since the lack of functional *B8R* and *B19R* genes has already been described in the attenuated MVA strain, which represents a safe and potent vaccine candidate (5, 7), we considered inactivation of these two VACV genes that antagonize the IFN system a possible attractive strategy for improving the safety and immunogenicity properties of recombinants based on the NYVAC strain.

We recently reported the generation and *in vitro* characterization of an improved NYVAC-C recombinant expressing the HIV-1 Env and Gag-Pol-Nef antigens from clade C, by deletion of the viral gene *B19R* (NYVAC-C-ΔB19R) (24, 42). In contrast to the parental NYVAC-C, the deletion mutant NYVAC-C-ΔB19R induced in human DCs enhanced IFN-α production, maturation, and expression of IFN-induced pathways and IFN-regulated transcription factors as well as multiple inflammatory cytokines. Here we have extended the earlier results by evaluating *in vivo* the effect of the deletion of the *B19R* and/or *B8R* gene on the immunogenicity of NYVAC-C. We asked to what extent the viral inhibitors of the IFN system impact the capacity of the NYVAC vector to activate specific adaptive and memory immune responses to HIV-1 antigens in a mouse model.

MATERIALS AND METHODS

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

Cells and viruses. Cells were maintained in a humidified air–5% CO_2 atmosphere at 37°C. Primary chicken embryo fibroblast (CEF) cells and African green monkey kidney cells (BSC-40) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The poxvirus strains used in this work included the genetically attenuated vaccinia virus-based vector NYVAC-WT 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 (17), used as the parental vector for the generation of the different deletion mutants. All viruses were grown in primary CEF cells similarly purified through two 45% (wt/vol) sucrose cushions, and the virus titers were determined by immunostaining in a plaque assay in monkey kidney BSC-40 cells as previously described (43). The titer determinations of the different viruses were performed at least three times.

Construction of plasmid transfer vector pGem-RG-B8R wm. The plasmid transfer vector pGem-RG-B8R wm, used for the construction of the recombinant virus NYVAC-C-ΔB8R, with the *B8R* open reading frame (ORF) deleted, was obtained by sequential cloning of five DNA fragments containing dsRed2 and rsGFP genes and *B8R* recombination flanking sequences into the plasmid pGem-7Zf(-) (Promega). The dsRed2 gene under the control of the synthetic early/late promoter was amplified by PCR from plasmid pG-dsRed2 with oligonucleotides Red2-B (5′-GAACTAGGATCCTAACTCGAGAAA-3′; BamHI site underlined) and Red2-N (5′-ATTAGTATGCATTTATTTATTTAGG-3′; NsiI site underlined) (785 bp), digested with BamHI and NsiI, and inserted into BamHI/NsiI-digested pGem-7Zf(-) to generate pGem-Red wm (3,740 bp). The rsGFP gene under the control of the synthetic early/late promoter was amplified by PCR from plasmid pG-dsRed2 with oligonucleotides GFP-X (5′-CGTTGGTCTAGAGAGAAAAATTG-3′; XbaI site underlined) and GFP-E (5′-CTATAGAATTCTCAAGCTATGC-3′; EcoRI site underlined) (832 bp), digested with XbaI and EcoRI, and inserted into plasmid pGem-Red wm previously digested with XbaI and EcoRI to obtain pGem-Red-GFP wm (4,540 bp). The NYVAC genome was used as the template to amplify the left flank of the *B8R* gene (358 bp) with oligonucleotides LFB8R-AatII-F (5′-TTTTTTGACGTCATTGACTCGTCTACTATTC-3′; AatII site underlined) and LFB8R-XbaI-R (5′-TTTTTTTCTAGATGGTGTTGTTTGTTATTTG-3′; XbaI site underlined). This left flank was digested with AatII and XbaI and cloned into plasmid pGem-Red-GFP wm previously digested with the same restriction enzymes to generate pGem-RG-LFsB8R wm (4,865 bp). The repeated left flank of *B8R* gene (358 bp) was amplified by PCR from the NYVAC genome with oligonucleotides LFB8R′-EcoRI-F (5′-TTTTTTGAATTCATTGACTCGTCTACTATTC-3′; EcoRI site underlined) and LFB8R′-ClaI-R (5′-TTTTTTATCGATTGGTGTTGTTTGTTATTTG-3′; ClaI site underlined), digested with EcoRI and ClaI, and inserted into EcoRI/ClaI-digested pGem-RG-LFsB8R wm to generate pGem-RG-LFdB8R wm (5,182 bp). The right flank of the *B8R* gene (367 bp) was amplified by PCR from the NYVAC genome with oligonucleotides RFB8R-ClaI-F (5′-TTTTTTATCGATCTAATTTTTATTAATGATAC-3′; ClaI site underlined) and RFB8R-BamHI-R (5′-TTTTTTGGATCCAAACAGCGGACACATTGC-3′; BamHI site underlined), digested with ClaI and BamHI, and inserted into ClaI/BamHI-digested pGem-RG-LFdB8R wm. The resulting plasmid pGem-RG-B8R wm (5,519 bp; see Fig. S1 in the supplemental material) was confirmed by DNA sequence analysis and found to direct the deletion of the *B8R* gene from the NYVAC-C genome.

The plasmid transfer vector pGem-RG-B19R wm, used for the construction of the recombinant viruses NYVAC-C-ΔB19R and NYVAC-C-ΔB8R/ΔB19R with *B19R* deleted or with both *B8R* and *B19R* genes deleted, respectively, has been previously described (24).

Construction of deletion mutants NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, and NYVAC-C-ΔB8R/ΔB19R. The deletion mutant NYVAC-C-ΔB8R was constructed using dsRed2 and rsGFP as markers. A total of 3×10^6 BSC-40 cells were infected with 0.01 PFU/cell of NYVAC-C and transfected 1 h later with 6 μg DNA of plasmid pGem-RG-B8R wm by using Lipofectamine (Invitrogen, San Diego, CA) according to the manufacturer's recommendations. After 48 h postinfection, the cells were harvested, lysed by freeze-thaw cycling, sonicated, and used for recombinant virus screening. A deletion mutant was selected from progeny virus by consecutive rounds of plaque purification in BSC-40 cells, during which plaques were screened for Red2/green fluorescent protein (GFP) fluorescence. In the first two passages, viruses from selected plaques expressed both fluorescent proteins, while in the next two passages viral progeny from selected plaques expressed only one fluorescent marker (Red2 or GFP). In the last two passages (six passages in total), viruses from selected plaques did not express any marker, due to the loss of the fluorescent marker by homologous recombination within the repeated flanking DNA sequences.

The construction of the deletion mutant NYVAC-C-ΔB19R has been previously described (24).

The double deletion mutant NYVAC-C-ΔB8R/ΔB19R was constructed using the same procedure described above for the NYVAC-C-ΔB8R generation. A total of 3×10^6 BSC-40 cells were infected with 0.01 PFU/cell of NYVAC-C-ΔB8R and transfected 1 h later with 6 μg DNA of plasmid pGem-RG-B19R wm by using Lipofectamine (Invitrogen, San Diego, CA). At 48 h postinfection, the cells were harvested, lysed by freeze-thaw cycling, sonicated, and used for recombinant virus screening.

PCR analysis of deletion mutants. To test the correct generation and purity of the deletion mutants, viral DNA was extracted from BSC-40 cells that were mock infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. Cell membranes were disrupted 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 LFB8R-AatII-F and LFB8R-BamHI-R spanning *B8R*-flanking regions were used for PCR analysis of the *B8R* locus. Primers LFB19R-AatII-F and LFB19R-BamHI-R spanning *B19R*-flanking regions were used for PCR analysis of the *B19R* locus. The amplification reactions were carried out with Platinum *Taq* DNA polymerase (Invitrogen, San Diego, CA).

Expression of HIV-1 proteins gp120 and GPN. To test the correct expression of HIV-1 antigens by the deletion mutants, monolayers of BSC-40 cells were mock infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. At 24 h postinfection, cells were lysed in Laemmli buffer, and cell extracts were fractionated by 8% SDS-PAGE and analyzed by Western blotting with a polyclonal anti-gp120 antibody (diluted 1:3,000; Centro Nacional de Biotecnología) or polyclonal anti-Gag p24 serum (ARP 432, diluted 1:1,000; NIBSC, Centralised Facility for AIDS Reagents, United Kingdom) to evaluate the expression of gp120 and Gag, Pol, and Nef (GPN) proteins, respectively. An anti-rabbit horseradish peroxidase-conjugated antibody (Sigma) diluted 1:5,000 was used as secondary antibody. The immunocomplexes were detected by enhanced chemiluminescence (ECL; GE Healthcare).

Analysis of virus growth. To determine virus growth profiles, monolayers of CEF cells grown in 12-well tissue culture plates were infected in duplicate at 0.01 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. Following virus adsorption for 60 min at 37°C, the inoculum was removed. The infected cells were washed once with DMEM without serum and incubated with fresh DMEM containing 2% FCS at 37°C in a 5% CO_2 atmosphere. At different times postinfection (0, 24, 48, and 72 h), cells were removed by scraping (lysates at 5×10^5 cells/ml), freeze-thawed three times, and briefly sonicated. Virus titers in cell lysates were determined by crystal violet staining in BSC-40 cells.

DNA vectors. The two DNA constructs expressing the HIV-1 $_{CN54}$gp120 (pcDNA-$_{CN54}$gp120) and HIV-1 $_{CN54}$Gag-Pol-Nef fusion protein (pcDNA-$_{CN54}$GPN) have been previously described (17). Plasmids were purified using Maxi-prep purification kits (Qiagen, Hilden, Germany) and diluted for injection in endotoxin-free phosphate-buffered saline (PBS).

Peptides. The HIV-1 peptide pools Gag-1, Gag-2, Env-1, Env-2, GPN-1, GPN-2, GPN-3, and NEF were provided by the EuroVacc Foundation and were previously described (17). They spanned the HIV-1 Env, Gag, Pol, and Nef antigens from clade C included in the immunogens as consecutive 15-mers overlapping by 11 amino acids. For immunological analyses, we grouped the pools as follows: Env pool (Env-1 plus Env-2), Gag pool (Gag-1 plus Gag-2), and GPN pool (GPN-1 plus GPN-2 plus GPN-3 plus NEF). The VACV $E3_{140-148}$ and $F2(G)_{26-34}$ peptides, previously described as immunodominant epitopes in BALB/c mice (50), were used to detect the antivector cellular immune response.

Mouse immunization schedule. BALB/c mice were purchased from Harlan. A DNA prime/poxvirus boost immunization protocol was performed to assay the immunogenicity of the different deletion mutants. Groups of animals ($n = 8$) received 100 μg of DNA-C (50 μg of pcDNA-$_{CN54}$gp120 plus 50 μg of pcDNA-$_{CN54}$GPN) by the intramuscular (i.m.) route and 2 weeks later received an intraperitoneal (i.p.) inoculation of 1×10^7 PFU of the corresponding virus. Animals primed with sham DNA (DNA-ϕ) and boosted with the nonrecombinant NYVAC-WT were used as a control group. At 10 and 53 days after the last immunization, 4 mice in each group were sacrificed and spleens were processed for intracellular cytokine staining (ICS) assays to measure the adaptive and memory immune responses against HIV-1 antigens, respectively. Two independent experiments were performed for the different groups.

ICS assay. The magnitude, polyfunctionality, and phenotypes of the HIV-1-specific T cell responses were analyzed by ICS. After an overnight rest, 5×10^6 splenocytes (depleted of red blood cells) were stimulated during 6 h in complete RPMI 1640 medium containing 1 μl/ml Golgiplug (BD Biosciences) and 5 μg/ml of the different HIV-1 peptide pools. At the end of the stimulation period, cells were washed, stained for the surface markers, permeabilized (Cytofix/Cytoperm kit; BD Biosciences), and stained intracellularly with the appropriate fluorochromes. For functional analyses, the following fluorochrome-conjugated antibodies were used: CD3-fluorescein isothiocyanate (FITC), CD4-Alexa 700, CD8-peridinin chlorophyll protein or CD8-V500, IL-2–phycoerythrin (PE) or IL-2–allophycocyanin (APC), IFN-γ–APC or IFN-γ–PE-Cy7, and tumor necrosis factor alpha (TNF-α)–PE-Cy7 or TNF-α–PE. In addition, for phenotypic analyses the following antibodies were used: CD62L-FITC and CD44-SPRD. Dead cells were excluded by using the violet LIVE/DEAD stain kit (Invitrogen). All antibodies were from BD Biosciences. Cells were acquired using an LSRII flow cytometer (BD Immunocytometry Systems). Analyses of the data were performed using the FlowJo software version 8.5.3 (Tree Star, Ashland, OR). The number of lymphocyte-gated events ranged between 10^5 and 10^6. After gating, Boolean combinations of single functional gates were then 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. Background responses detected in negative-control samples were subtracted from those detected in stimulated samples for every specific functional combination.

ELISPOT assay. The VACV-specific cellular immune response was evaluated in a fresh IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay as previously described (28). The E3 and F2(G) peptides were resuspended in RPMI 1640 supplemented with 10% FCS and added to the cells at a final concentration of 5 μg/ml.

Antibody measurements by ELISA. Binding antibodies to Env and vaccinia virus proteins in serum were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (17). Serum samples from naïve and immunized mice were serially 2-fold diluted in duplicate and reacted against 2 μg/ml of the recombinant 97CN54 gp140 purified protein (clade C; kindly provided by Simon A. Jeffs, Imperial College London, London, United Kingdom). The antibody titer of Env-specific IgG was defined as the last dilution of serum that resulted in 3 times the mean optical density at 450 nm of the naïve control. To evaluate the anti-VACV humoral response, serum from individual mice was reacted at a 1:100 dilution in triplicate against 10 μg/ml of extract from BSC-40 cells infected (5 PFU/cell) for 24 h with VACV WR.

Data analysis and statistics. For the statistical analysis of ICS data, we used a novel approach that corrected measurements for the medium response (RPMI medium) and at the same time allowed the calculation of confidence intervals and *P* values of hypothesis tests (15, 34). The background for the different cytokines in the unstimulated controls never exceeded 0.05%. The data analysis program Simplified Presentation of Incredibly Complex Evaluations (SPICE, version 4.1.5; Mario Roederer, Vaccine Research Center, NIAID, NIH) was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. Background values were subtracted from all values used to allow analysis of proportionate representation of responses.

RESULTS

Generation and *in vitro* characterization of NYVAC-C deletion mutants. NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, and NYVAC-C-ΔB8R/ΔB19R deletion mutants were generated as described in Materials and Methods, using as parental virus the recombinant NYVAC-C, which expresses the HIV-1 Env, Gag, Pol, and Nef antigens from clade C (17). The correct generation and purity of each deletion mutant were confirmed by PCR using primers for

the *B8R* or *B19R* locus, showing correct deletion of the viral gene (Fig. 1A). Analysis by Western blotting verified that the deletion mutants expressed the HIV-1 proteins gp120 and GPN at the same levels as their parental virus, NYVAC-C (Fig. 1B). Moreover, analysis by immunostaining showed that all virus plaques had immunoreactivities to both anti-WR and anti-gp120 antibodies similar to the parental NYVAC-C (data not shown), demonstrating the stability of the viruses. To determine if single or double deletions of the *B8R* and *B19R* genes affected virus replication in cell cultures, we compared the growth kinetics in CEF cells of all deletion mutants with their parental virus, NYVAC-C. Figure 1C shows that the kinetics of growth were similar between parental and deletion mutants. This indicates that when deleted individually or in double, type I (B19) and type II (B8) IFN binding proteins are not required for virus replication in cultured cells. To determine if deletion of *B8R* and/or *B19R* would have an effect on biomarkers downstream of IFN binding to its receptor, we analyzed the phosphorylation levels of STAT1 in extracts from human peripheral blood mononuclear cells infected (5 PFU/cell) for 4 h with the parental virus or a deletion mutant. In contrast with NYVAC-WT, NYVAC-C, or NYVAC-C-ΔB8R, viruses lacking *B19R* or with a double deletion had high levels of p-STAT1, indicating activation of this signaling pathway (see Fig. S1B in the supplemental material).

Deletion of the viral gene *B8R* and/or *B19R* in NYVAC-C induced high, broad, and polyfunctional HIV-1-specific T cell adaptive immune responses. To assay *in vivo* the effects of the single and double deletions of *B8R* and *B19R* vaccinia virus genes in the genome of the NYVAC-C recombinant, we analyzed the HIV-1-specific immune responses elicited in mice by using a DNA prime/poxvirus boost approach. BALB/c mice, 4 in each group, were immunized according to the schedule shown in Fig. 2A. Animals received 100 μg of DNA-C (50 μg of pcDNA-$_{CN54}$gp120 plus 50 μg of pcDNA-$_{CN54}$GPN) by the i.m. route followed by an i.p. injection of 1×10^7 PFU of recombinant viruses. Adaptive T cell immune responses were measured at day 25 by polychromatic ICS assay after the stimulation of splenocytes with a panel of 464 peptides (15-mers overlapping by 11 amino acids) grouped in three pools: Env (112 peptides), Gag (121 peptides), and GPN (231 peptides). The peptides encompassed the Env, Gag, Pol, and Nef proteins of HIV-1 and were designed based on the sequence of the immunogens expressed by NYVAC-C. Animals primed with sham DNA (DNA-ϕ) and boosted with the nonrecombinant NYVAC-WT were used as a control group. As shown in Fig. 2B, in all the immunization groups the magnitude of the HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses, determined as the sum of the individual responses obtained for Env, Gag, and GPN peptide pools, was significantly higher than that obtained in the control group, DNA-ϕ/NYVAC-WT ($P < 0.05$). The $CD4^+$ T cell responses were mainly directed against the Env pool, with no differences between the groups boosted with the NYVAC-C deletion mutants and the group boosted with the parental NYVAC-C. In contrast, the $CD8^+$ T cell responses were higher in magnitude and were significantly higher in the groups boosted with the NYVAC-C deletion mutants than in the group boosted with the parental NYVAC-C. Clearly, the deletions induced a significant enhancement in the magnitude of the $CD8^+$ T cell responses against the Env pool ($P < 0.005$), whereas the anti-GPN responses were not significantly affected. No specific $CD4^+$ or $CD8^+$ T cell responses were detected against the Gag pool. Representative functional profiles of Env-induced $CD8^+$ T cell responses are shown in Fig. 2C. Similar findings were observed in two independent experiments.

The quality of a T cell response can be characterized in part by the pattern of cytokine production. On the basis of the analysis of IL-2, TNF-α, and IFN-γ secretion, seven distinct HIV-1-specific $CD4^+$ and $CD8^+$ T cell populations were identified (Fig. 3). The percentages of cells producing cytokines obtained in the DNA-ϕ/NYVAC-WT control populations were subtracted for all the groups in order to remove the nonspecific responses detected as background. Vaccine-induced $CD4^+$ T cell responses were highly polyfunctional in all the immunization groups, with more than 60% of $CD4^+$ T cells exhibiting two or three functions. $CD4^+$ T cells producing IL-2, TNF-α, and IFN-γ, IL-2 and TNF-α, or only IL-2 were the most representative populations induced by the parental NYVAC-C and the deletion mutants, although the percentages of cells that produced cytokines were low (Fig. 3A). The HIV-1-specific $CD8^+$ T cell responses were also highly polyfunctional (proportions of cells producing the different numbers of cytokines were similar in all the immunization groups). $CD8^+$ T cells producing IL-2, TNF-α, and IFN-γ, IFN-γ and TNF-α, or only IFN-γ were the most representative populations induced by the parental NYVAC-C and the deletion mutants, but the absolute frequencies of each population were significantly higher in groups boosted with the NYVAC-C-ΔB8R and NYVAC-C-ΔB8R/ΔB19R deletion mutants than with the parental NYVAC-C (Fig. 3B). Overall, these results indicated that single and/or double deletion of the viral genes *B8R* and *B19R* improved the magnitude and quality of HIV-1-specific adaptive T cell immune responses.

Impact of single and/or double deletion of the viral genes *B8R* and *B19R* on the $CD8^+$ T cell memory phase of the immune response. Phenotypic analysis of memory vaccine-induced T cell responses was carried out 53 days after the last immunization in a polychromatic ICS assay. Splenocytes from immunized mice were stimulated with the HIV-1 peptide pools Env, Gag, and GPN for 6 h and stained with specific antibodies to identify T cell lineage (CD4 and CD8) and responding cells (IL-2, IFN-γ, and TNF-α), as well as memory stages (CD44 and CD62L).

The magnitudes of the memory HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses, determined as the sum of the individual responses obtained for Env, Gag, and GPN peptide pools, were significantly higher in all the groups boosted with the parental or the deletion NYVAC-C mutants than in the control group, DNA-ϕ/NYVAC-WT ($P < 0.05$) (Fig. 4A). The contributions of CD4 and CD8 T cell subsets to the HIV-1-specific responses were similar in the groups of animals boosted with NYVAC-C and NYVAC-C-ΔB19R, whereas in groups boosted with NYVAC-C-ΔB8R and NYVAC-C-ΔB8R/ΔB19R the response was mainly mediated by the CD8 T cells. All deletion mutants and the parental NYVAC-C induced a similar pattern of HIV-1-specific $CD4^+$ T cell memory responses (with preference toward Env). However, the patterns of $CD8^+$ T cell memory responses were different between the vectors: DNA-C/NYVAC-C, DNA-C/NYVAC-C-ΔB19R, and DNA-C/NYVAC-C-ΔB8R/ΔB19R induced a higher percentage of GPN-specific $CD8^+$ T cell memory responses, while DNA-C/NYVAC-C-ΔB8R induced preferentially Env-specific $CD8^+$ T cell memory responses. Significantly, the magnitude of GPN-specific $CD8^+$ T cells was the highest for the group DNA-C/NYVAC-C-ΔB8R/ΔB19R, followed by DNA-C/NYVAC-C-ΔB19R.

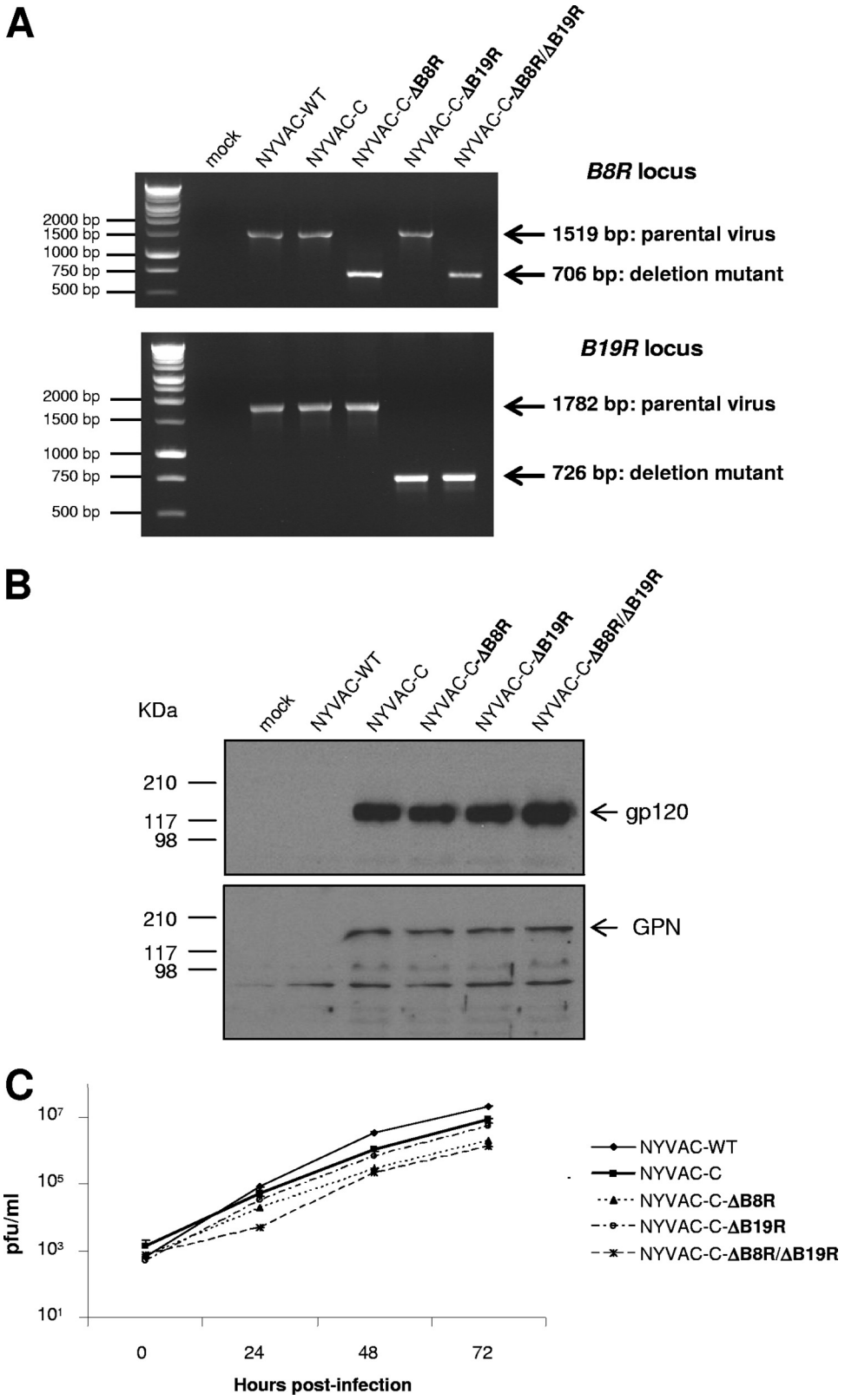

FIG 1 *In vitro* characterization of NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, and NYVAC-C-ΔB8R/ΔB19R deletion mutants. (A) PCR analysis. Viral DNA was extracted from BSC-40 cells mock infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. Primers spanning *B8R*- or *B19R*-flanking regions were used for PCR analysis of the *B8R* or *B19R* locus, respectively. (B) Expression of HIV-1 antigens gp120 and GPN. BSC-40 cells were mock infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. At 24 h postinfection, cells were lysed in Laemmli buffer, and cell extracts were fractionated by 8% SDS-PAGE and analyzed by Western blotting with rabbit polyclonal anti-gp120 antibody or polyclonal anti-Gag p24 serum. (C) Replication in CEF cells. CEF cells were infected at 0.01 PFU/cell with NYVAC-WT, NYVAC-C, NYVAC-C-ΔB8R, NYVAC-C-ΔB19R, or NYVAC-C-ΔB8R/ΔB19R. At different times postinfection (0, 24, 48, and 72 h), cells were harvested and virus titers in cell lysates were determined by crystal violet staining in BSC-40 cells.

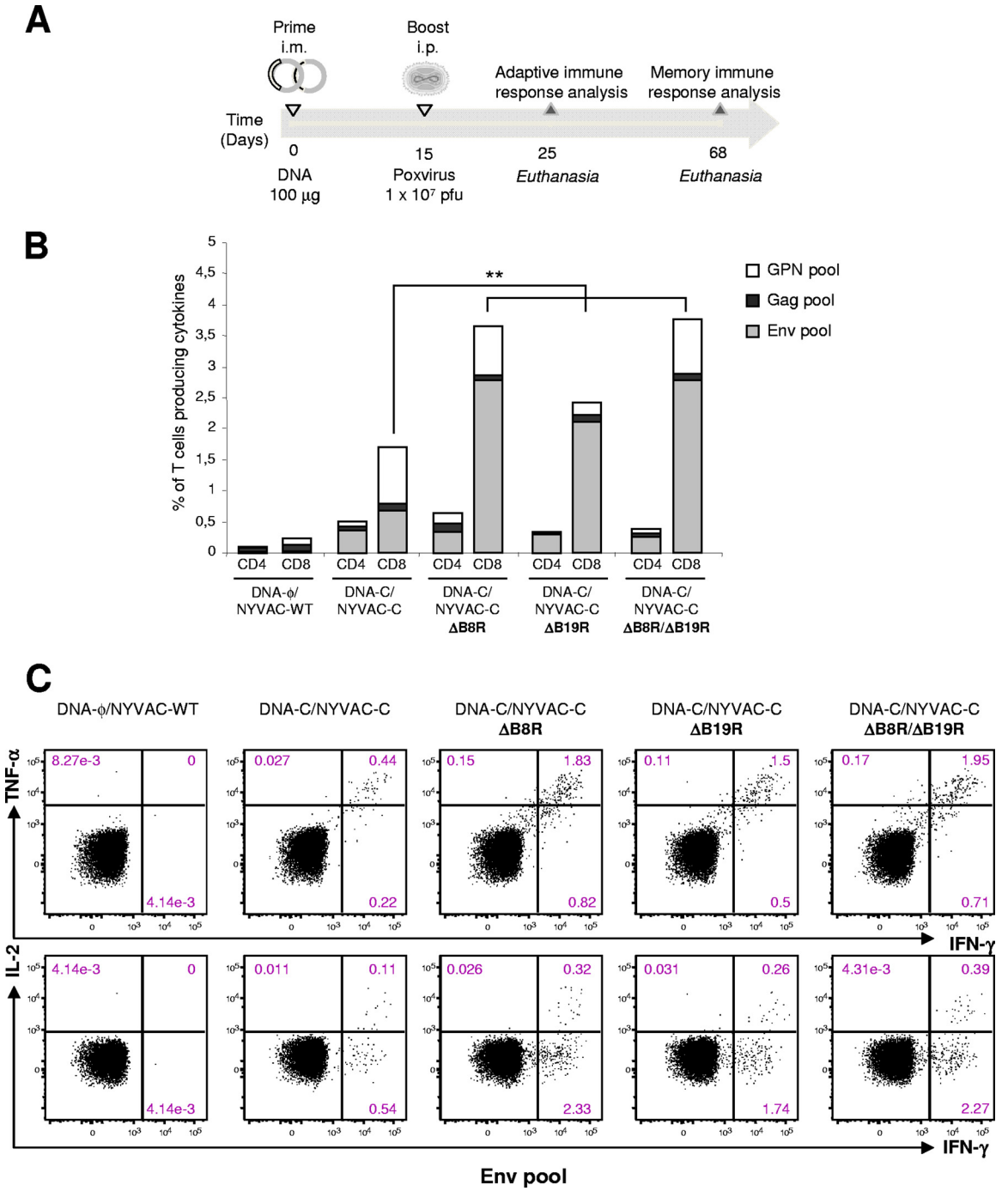

FIG 2 Adaptive HIV-1-specific immune responses elicited by the deletion mutants. (A) Schematic diagram showing the vaccination schedule followed in the study and the immunogenicity end points. (B) Magnitude of vaccine-specific $CD4^+$ and $CD8^+$ T cells. HIV-1-specific $CD4^+$ and $CD8^+$ T cells were measured 10 days after the last immunization in an ICS assay following stimulation with the different HIV-1 peptide pools. The values represent the sum of the percentages of T cells secreting IFN-γ and/or TNF-α and/or IL-2 against Env plus Gag plus GPN peptide pools. Background values were subtracted from all data. (C) Flow cytometry profiles of vaccine-induced $CD8^+$ T cell responses against the Env peptide pool. **, $P < 0.005$ (compared to the DNA-C/NYVAC-C immunization group).

At day 53, the deletion mutants, but not NYVAC-C, changed the contraction of responding $CD8^+$ T cells. Contraction of the $CD8^+$ T cell pool, determined as the ratio of HIV-1-positive cells at day 10 postboost and day 53 postboost, was reduced in the groups of animals inoculated with the deletion mutants, in comparison with animals immunized with the parental NYVAC-C (Fig. 4B).

We also determined the phenotype of the memory responses by measuring the expression of CD62L and CD44 in the HIV-1-specific T cells. We chose to monitor the expression of CD62L,

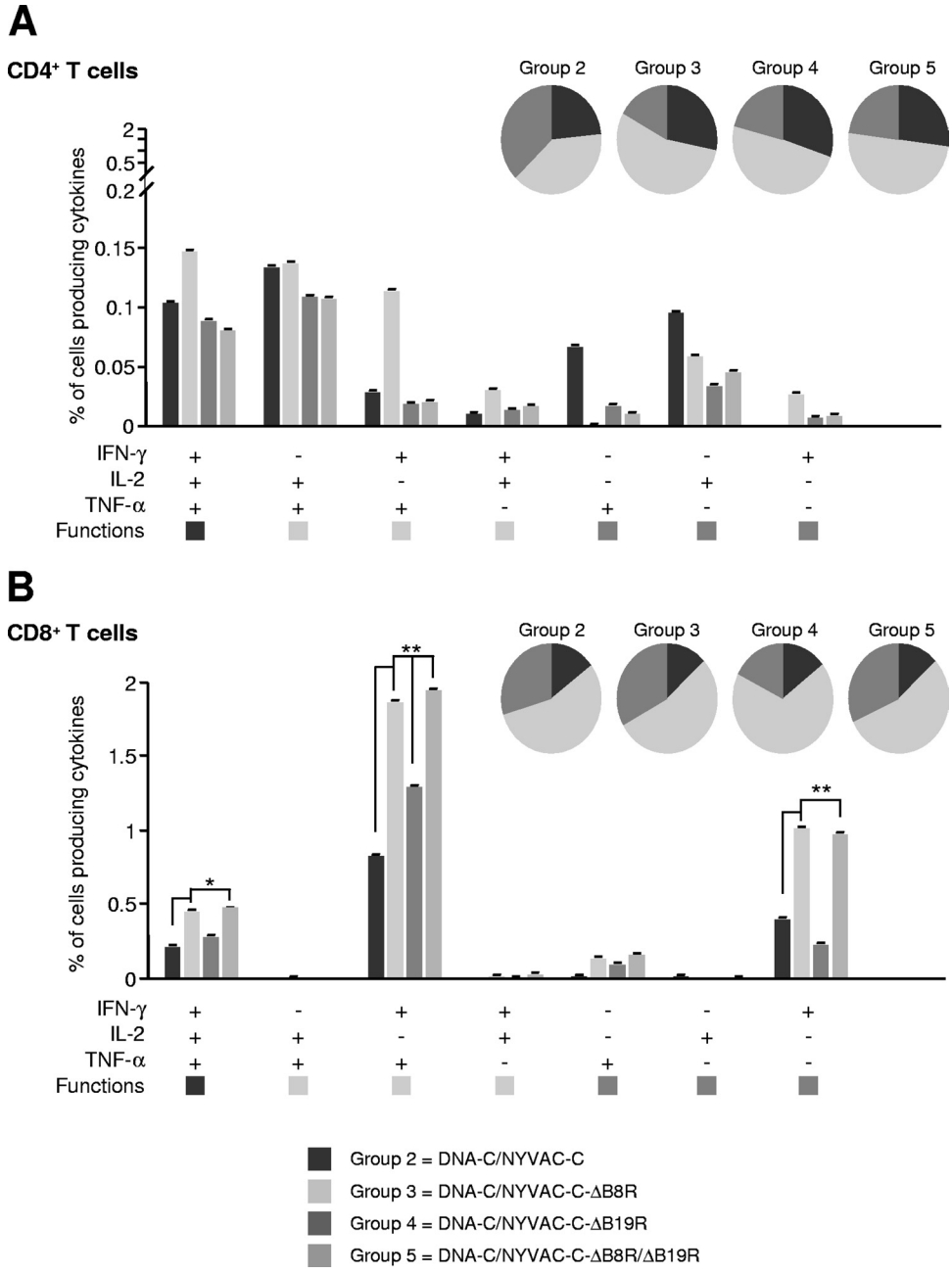

FIG 3 Functional profiles of adaptive HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses in the different immunization groups. All the possible combinations of the responses are shown on the *x* axis, whereas the percentages of the functionally distinct cell populations within the total CD4 (A) or CD8 (B) T cell populations 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 group DNA-*ϕ*/NYVAC-WT were subtracted for all populations. *, $P < 0.05$; **, $P < 0.005$ (compared to the DNA-C/NYVAC-C immunization group).

because it is a key marker that segregates the effector and central memory T cell subsets in combination with CD44, which is expressed at high levels in all effector and central memory T cells, but not in naïve T cells (51). Thus, the effector memory T cells (TEM) have a $CD44^{high}$ $CD62L^-$ phenotype, whereas the central memory T cells (TCM) are $CD44^{high}$ $CD62L^+$. For CD4 T cells, the Env-specific memory responses elicited by NYVAC-C and the deletion mutants were predominantly TEM (see Fig. S2 in the supplemental material). Similarly, for CD8 T cells the HIV-1-specific memory responses induced by NYVAC-C and the deletion mutants were TEM, but some differences were observed between the groups. After NYVAC-C boost, 69.2% and 53.1% of the $CD8^+$ T cells against Env and GPN pools, respectively, were TEM, whereas the single or double deletion of *B8R* and *B19R* increased these populations up to 90% (Fig. 4C).

To analyze the quality of the HIV-1-specific memory responses, we evaluated the IL-2, TNF-α, and IFN-γ secretion levels in both CD4 and CD8 T subsets. Vaccine-induced $CD4^+$ T cell memory responses were similarly polyfunctional in all the immunization groups. $CD4^+$ T cells producing IL-2 and TNF-α or only

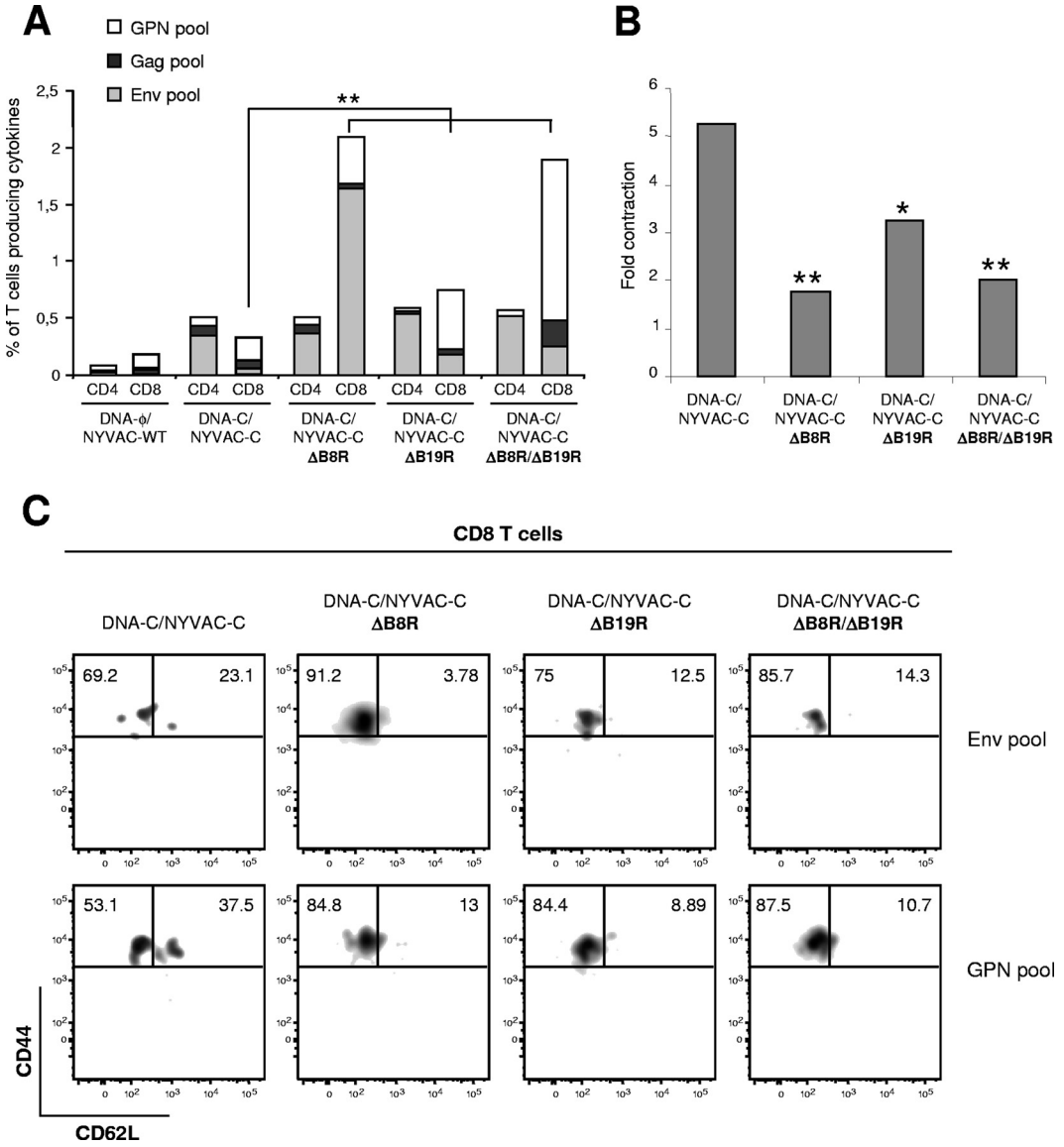

FIG 4 Memory responses to HIV-1 antigens elicited by the deletion mutants. (A) Magnitude of vaccine-specific $CD4^+$ and $CD8^+$ T cells. The HIV-1-specific CD4 and CD8 T cells were measured 53 days after the last immunization by ICS assay following stimulation with the different HIV-1 peptide pools. The values represent the sum of the percentages of T cells secreting IFN-γ and/or TNF-α and/or IL-2 against Env plus Gag plus GPN peptide pools. Background percentages were subtracted from all data. (B) Fold contraction of the frequency of HIV-1-specific CD8 T cells following boost. The fold contraction was calculated as the ratio of HIV-1-positive cells at day 10 postboost and day 53 postboost. (C) Phenotypic profiles of memory HIV-1-specific CD8 T cells. Representative fluorescence-activated cell sorting plots show the percentage of Env- and GPN-specific CD8 T cells with central memory (TCM; $CD44^{high}$ $CD62L^+$) or effector memory (TEM; $CD44^{high}$ $CD62L^-$) phenotype.

TNF-α were the most representative populations induced by the recombinant viruses (Fig. 5A). In contrast, the HIV-1-specific $CD8^+$ T cell memory responses were improved by the use of the deletion mutants, in terms of both magnitude and polyfunctionality. The percentage of $CD8^+$ T cells producing IL-2, TNF-α, and IFN-γ, IFN-γ and TNF-α, or only TNF-α were significantly increased in animals boosted with the deletion mutants (Fig. 5B). Overall, these results revealed that single and/or double deletion of the viral genes *B8R* and *B19R* impacts the $CD8^+$ T cell memory phase of the immune response, changing the contraction phase, memory cell differentiation, the magnitude, and the polyfunctionality pattern.

Deletion of the viral genes *B8R* and/or *B19R* in NYVAC-C does not affect the anti-HIV-1 gp120 humoral response. Since cells infected with NYVAC-C release monomeric gp120 (17), we evaluated the impact of the deletion of viral genes *B8R* and/or *B19R* on the humoral response at days 25 and 68. We quantified by using ELISA the Env-specific IgG titers against the purified gp140 protein from the HIV-1 isolate CN54 (clade C) (Fig. 6). In all the groups boosted with the deletion mutants, the levels of anti-gp140 antibodies were similar to those obtained in animals immunized with the parental NYVAC-C at both times assayed (days 25 and 68), indicating that deletion of the viral genes *B8R* and/or *B19R* has no effect on the humoral immune response induced in mice.

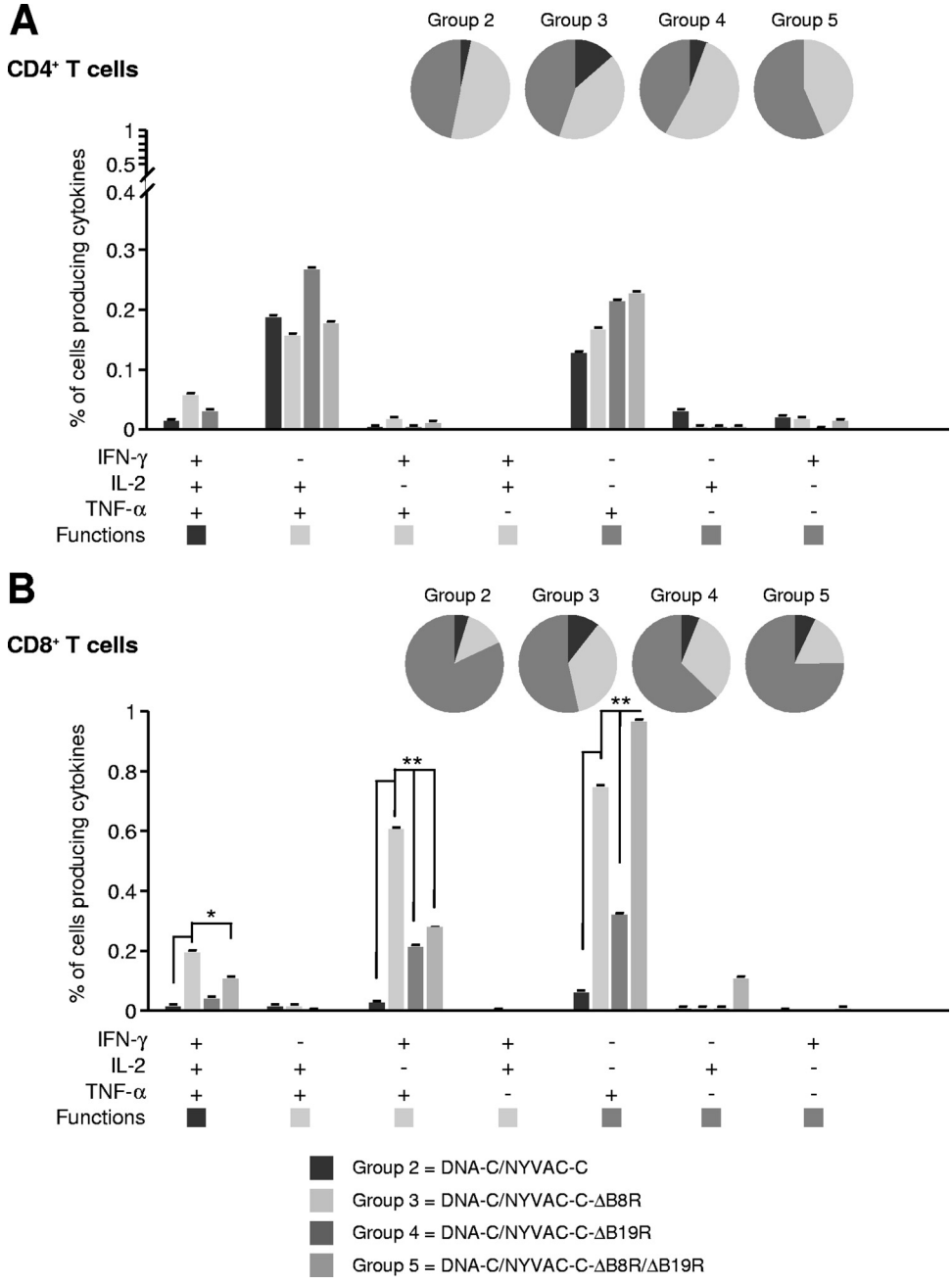

FIG 5 Functional profile of memory HIV-1-specific $CD4^+$ and $CD8^+$ T cell responses. All the possible combinations of the responses are shown on the *x* axis, whereas the percentage of the functionally distinct cell populations within the total CD4 (A) or CD8 (B) T cell populations are shown on the *y* axis. Responses are grouped and color coded on the basis of the number of functions. The nonspecific responses obtained in the control group (DNA-ϕ/NYVAC-WT) were subtracted for all populations. *, $P < 0.05$; **, $P < 0.005$ (compared to response of DNA-C/NYVAC-C immunization group).

Antivector immune responses. Vaccine-induced antivector T cell responses were assessed in a fresh IFN-γ ELISPOT assay against the E3 and F2 epitopes at days 25 and 68. At day 25, the number of IFN-γ-secreting cells reacting against both peptides was similar in all the immunization groups (Fig. 7A). The same result was obtained at day 68 (data not shown). In addition, we evaluated the antivector humoral responses in an ELISA. There were not significant differences in the anti-VACV binding antibodies between the groups at any time assessed (Fig. 7B). Similar findings were observed in two independent experiments. Altogether, these results indicate that deletion of *B8R* and/or *B19R* in the NYVAC-C genome does not affect the cellular or humoral vaccine-induced antivector responses in mice.

DISCUSSION

Poxviruses offer attractive and unique properties for the generation of highly stable recombinant vectors as vaccine candidates; hence, they are increasingly used in the prevention and treatment

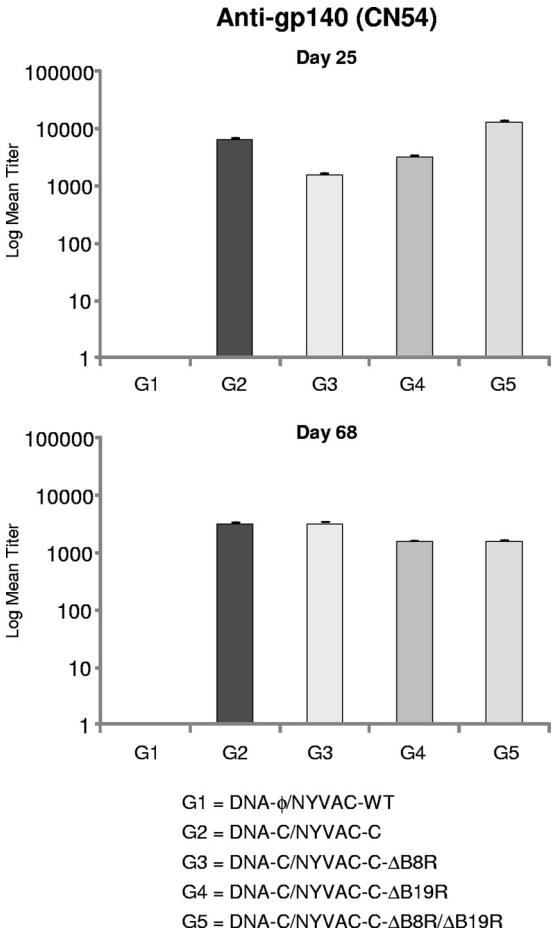

FIG 6 Humoral immune responses elicited by the deletion mutants against HIV-1 gp140 protein. Levels of Env-specific IgG binding antibodies were measured in serum from naïve and immunized mice at days 25 and 68. The values represent the mean antibodies titer for each group.

of emergent infectious diseases and cancer, in particular, those candidates based on the highly attenuated ALVAC, MVA, and NYVAC strains (18, 35). Despite the safety and immunogenicity profiles exhibited by these attenuated VACV strains, more-efficient vectors that enhance the magnitude, breadth, polyfunctionality, and durability of the immune responses to exogenously expressed antigens are desirable. One approach to improve the vector immunogenicity is to remove from the viral DNA genes that antagonize host-specific immune responses. This strategy has being successfully used for MVA and NYVAC vectors by targeting several immunomodulatory genes (8, 10, 15, 16, 24, 42, 47). Since virus recognition and induction of IFNs are critical components of the innate immune system, here we evaluated in a mouse model how the single or double deletion of the viral genes *B8R* and *B19R* affected the immunogenicity of the HIV/AIDS vaccine candidate NYVAC-C.

The NYVAC-C recombinant expresses the HIV-1 gp120 and Gag-Pol-Nef proteins from clade C and has been extensively studied in preclinical studies (17, 30, 56) and clinical trials (6, 20, 27). In humans, the best responses to HIV-1 antigens were obtained when using the heterologous DNA-C prime/NYVAC-C boost approach (90% responders), while two NYVAC-C immunizations induced specific responses only in 40% of the vaccinees (6, 20). In our study, using the optimal DNA-C prime/NYVAC-C boost protocol, we observed in mice that deletion of *B19R* and/or *B8R* genes, encoding proteins acting as antagonists of type I and type II IFN-mediated antiviral host defenses from the NYVAC-C genome, markedly improved the magnitude and functional quality of adaptive and memory HIV-1-specific $CD8^+$ T cells without altering $CD4^+$ T cell or humoral anti-gp120 responses.

While priming with DNA has been shown to preferentially drive the immune response to $CD4^+$ T cells following boosting with NYVAC in both monkeys and humans (20, 30), it is significant that boosting with the NYVAC deletion mutants preferentially triggered $CD8^+$ T cell responses, highlighting the contribution of both the *B8R* and/or *B19R* genes in directing immune responses in DNA/NYVAC protocols. The differences observed between the HIV-1-specific $CD8^+$ T cell responses induced in animals boosted with the NYVAC-C deletion mutants might be attributed to the effects that these viruses have on DCs. It has been reported that $CD8^+$ T cells need various stimuli to become fully activated and to induce differentiation and proliferation. These stimuli can be divided into two categories: T cell receptor signals and environmental cues, including but not limited to DC activation and costimulation, CD4 help, and soluble inflammatory growth factors (41). We previously observed that deletion of *B19R* in NYVAC-C resulted in enhanced expression of IFN and IFN-induced genes, transcription factors, and inflammatory cytokines in both conventional and plasmacytoid DCs. In conventional DCs, these changes were associated with enhanced IFN-α production and enhanced expression of the costimulatory molecule CD86 (42). Furthermore, deletion of *B8R* and *B19R* from NYVAC-C resulted in a strong inflammatory response, as evidenced by the transcriptional upregulation of IFN and IFN-induced genes in DCs (24). All of these findings reveal that in the absence of the viral inhibitors of type I and/or type II IFNs, the NYVAC recombinants are more immunogenic because they are efficiently sensed by the DCs and other antigen-presenting cells (APCs), leading to the generation of an optimal environment for the activation and differentiation of high-quality HIV-1-specific $CD8^+$ T cells.

The efficacy of viral vaccines depends in part on the generation of a pool of potent memory T cells ready to expand rapidly upon reexposure to the antigen. Here we observed that the $CD8^+$ T cells induced after boosting with the deletion mutants underwent higher expansion and lower contraction than the $CD8^+$ T cells induced after the boost with the parental NYVAC-C, which resulted in a 2- to 5-fold increase in HIV-1-specific memory responses. The patterns of contraction exhibited by the $CD8^+$ T cells activated by the deletion mutants, and in particular by NYVAC-C-ΔB8R or NYVAC-C-ΔB8R/ΔB19R, were similar to those observed with homologous and heterologous immunizations with the adenovirus 5 (Ad5) vector (40), or after immunization with live attenuated influenza viruses that express altered NS1 protein, which is known to act as an inhibitor of host IFN responses (32), demonstrating the critical role of IFNs in the generation of an effective immune responses. It has been described that optimal priming of both CD8 and CD4 T cell responses involves direct signaling through the IFN type I receptor (IFN-α/βR) (11, 21, 25), while IFN type II receptor signaling in CD8 T cells is dispensable for expansion, contraction, and memory differentiation (46). Additionally, the $CD8^+$ T cells activated by the deletion mutants

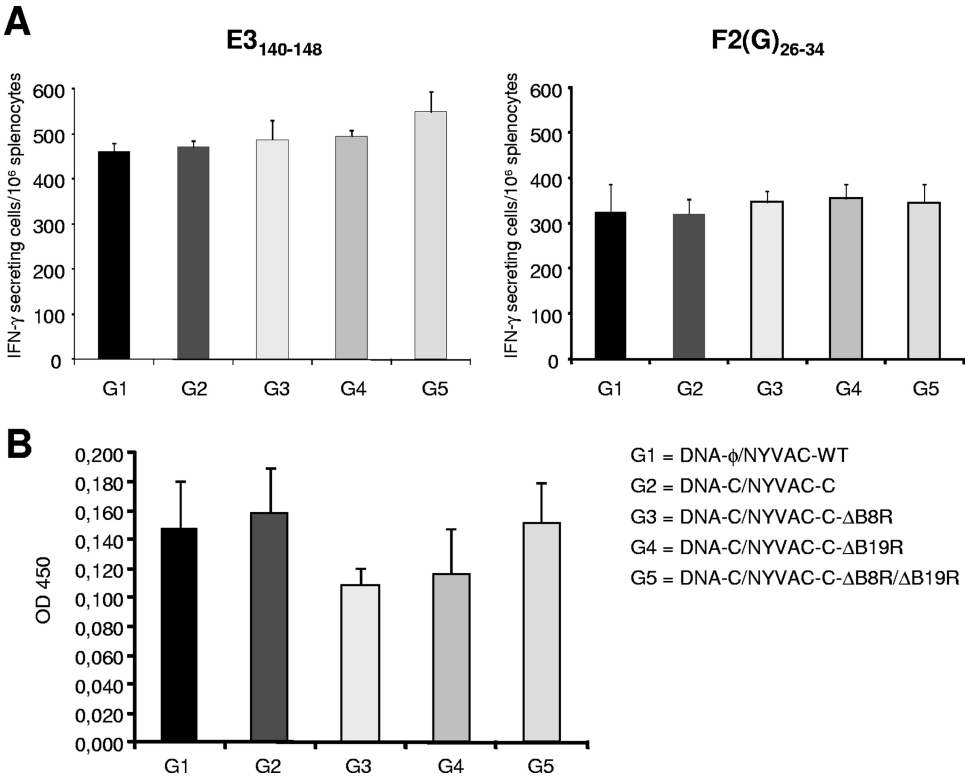

FIG 7 Antivector-induced immune responses. (A) Number of IFN-γ-secreting cells against the E3 and F2(G) peptide epitopes, measured by ELISPOT. (B) Anti-vaccinia virus binding antibodies. Serum from individual mice was evaluated by ELISA for specific anti-VACV antibodies.

enhanced differentiation to an effector memory phenotype and enhanced polyfunctionality compared with the $CD8^+$ T cells activated by the parental NYVAC-C. The relevance of the effector memory T cells on the early control of highly pathogenic simian immunodeficiency virus was recently described (19).

As we have shown in this study, deletion of *B8R* and/or *B19R* improves the HIV-1-specific CD8 T cell responses but does not affect the vaccine-induced antivector cellular or humoral immune responses. Similarly, in previous *in vitro* studies we observed that deletion of *B19R* from the NYVAC-C genome does not modify the anti-VACV-specific $CD8^+$ T cell responses compared to parental NYVAC-C, which were enhanced by replication-competent NYVAC-C-KC vectors (42). However, we cannot exclude the possibility that the antivector responses are modified by the deletions of *B8R* and/or *B19R*, since only a limited number of VACV epitopes were analyzed. It was previously described that deletion of *B8R* gene in MVA, although it is not functional, leads to an enhancement of boosted responses to other antigens in the boosting vector, and this is related to the timing of the expression of B8 relative to other antigens, such as an inserted OVA gene. Those authors chose *B8R*, as they were using C57BL/6 mice, in which B8 contains the immunodominant epitope (23). It is plausible that in our mouse model (BALB/c) the deletion of the early viral genes *B8R* and *B19R* in NYVAC also changed the antigenic dominance hierarchy, contributing to increased responses to the inserted HIV-1-derived antigens but without affecting the responses against the viral E3 or F2, two of the immunodominant peptides described for this haplotype. The apparent differences in immune responses between HIV-1 antigens and viral antigens by the *B8R* and/or *B19R* deletion mutants might be due to the combination of a limited replication capacity of the vector and epitope immunodominance.

Previous data concerning VACV vIFN-α/βR or vIFN-γR functions on vector immunogenicity after their blockade have not revealed any positive results (12, 22, 54). In those studies, the authors used different replication-competent VACV strains, such as WR (54), Lister (12), and Wyeth (22), which still retain the rest of the genes involved in VACV evasion of IFN signaling pathways. It is known that in addition to *B8R* and *B19R*, the products of the genes *E3L*, *K3L*, *H1L*, *C7L* and more recently *C6L* act within infected cells, blocking the IFN signaling cascade or IFN-induced antiviral state (16, 37, 52). The need for the virus to express so many different nonredundant viral inhibitors of host signaling cascades may be due to the pressure that the host innate immune system applies. It is plausible that the inhibition exerted by a single viral protein is not complete, particularly at early stages of infection, thus requiring the expression of several different factors targeting components of the same pathway, resulting in an additive effect. In the previous studies, it was evident that a single deletion in a replication-competent VACV strain did not improve the capacity of the host cells for priming effective antiviral responses. However, in our study we used as parental virus the attenuated NYVAC strain, which does not express the functions of the gene products of *K1L*, *C6L*, and *C7L*. As we have shown here, the deletion of *B8R* and/or *B19R* in the NYVAC-C genome significantly improved the magnitude and quality of the HIV-1-specific immune responses. If we consider to what extent the different deletions contribute to enhancing the immunogenicity of the HIV-1

antigens expressed from NYVAC-C in the mouse model, we can align the responses from higher to lower in the following order: for adaptive immune responses, the magnitude and polyfunctionality were NYVAC-C-ΔB8R/ΔB19R > ΔB8R > ΔB19R > parental. In the case of memory immune responses, the magnitude was higher in the order NYVAC-C-ΔB8R > ΔB8R/ΔB19R > ΔB19R > parental. For the effector phenotype and specifically for GPN responses, it followed the order NYVAC-C-ΔB8R/ΔB19R > ΔB19R > ΔB8R > parental. For the contraction phase and polyfunctionality, the order was NYVAC-C-ΔB8R > ΔB8R/ΔB19R > ΔB19R > parental. Clearly, the single and double deletions had an impact on the immune responses to HIV-1 antigens. Since there are lower affinities of vIFN-α/βR and vIFN-γR for mouse type I and type II IFNs than for other cell origins (3, 31, 48), our results suggest that in addition to acting as IFN receptor homologs, these viral proteins might exhibit still-undiscovered immunoregulatory activities. Thus, single or double deletion of the viral factors B8 and B19 targeting the IFN pathway might provide an effective strategy for the design of poxvirus-based vaccine candidates with improved immunogenicity.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Ministry of Science and Innovation of Spain (SAF2008-02036) and PTVDC/CAVD program with support from the Bill and Melinda Gates Foundation.

We are grateful to the EuroVacc Foundation for peptide pools of HIV-1 antigens from clade C.

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