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# Human Immunodeficiency Virus Type 1 Vpr Impairs Dendritic Cell Maturation and T-Cell Activation: Implications for Viral Immune Escape

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**Antigen presentation and T-cell activation are dynamic processes involving signaling molecules present in both APCs and T cells. Effective APC function and T-cell activation can be compromised by viral immune evasion strategies, including those of human immunodeficiency virus type 1 (HIV-1). In this study, we determined the effects of HIV-1 Vpr on one of the initial target of the virus, dendritic cells (DC), by investigating DC maturation, cytokine profiling, and CD8-specific T-cell stimulation function followed by a second signal. Vpr impaired the expression of CD80, CD83, and CD86 at the transcriptional level without altering normal cellular transcription. Cytokine profiling indicated that the presence of Vpr inhibited production of interleukin 12 (IL-12) and upregulated IL-10, whereas IL-6 and IL-1β were unaltered. Furthermore, DC infected with HIV-1 *vpr*$^+$ significantly reduced the activation of antigen-specific memory and recall cytotoxic-T-lymphocyte responses. Taken together, these results indicate that HIV-1 Vpr may in part be responsible for HIV-1 immune evasion by inhibiting the maturation of costimulatory molecules and cytokines essential for immune activation.**

Dendritic cells (DC) play a pivotal role in the generation of an effective immune response against incoming pathogens. As antigen-presenting cells (APCs), DC transport processed antigens from peripheral tissue to draining lymph nodes, where they present antigens to circulating T cells, initiating an antigen-specific T-cell response (5). DC typically respond to pathogens by undergoing a well-regulated maturation program requiring the transcriptional regulation of both costimulatory molecule and cytokine-chemokine genes necessary for antigen processing, presentation, and migration to lymphoid organs (25, 50). Because of their important role in inducing the antiviral response, many viruses and bacterial pathogens target DC and T cells (1, 2, 23, 30, 55). As part of their survival strategy, some microbial pathogens evade the host immune system by mimicking the proteins involved in host defense. Many viruses also code for proteins that counteract the host immune responses to infection. For example, poxviruses, cytomegalovirus, and herpesvirus encode homologue cytokine receptors, which can bind to the cognate cytokines and block their activities (23, 30). Viruses also encode host proteins and incorporate them into the virion, thus mimicking host defense molecules in order to escape immune surveillance (19, 55).

Upon exposure of human immunodeficiency virus type 1 (HIV-1) to the mucosal surface during transmission, different cells are exposed to the virus. Among these, immature DC, Langerhans cells, and resting T cells are the initial targets of HIV-1 infection that promote viral replication and dissemination via immune synapses (26, 28, 36, 48, 64). For example, immature DC, through viral engagement with C-type lectins, may be one of the first leukocytes to capture and replicate HIV-1 crossing a mucosal surface, thereby transmitting virus to nearby CD4$^+$ T cells for amplification through DC and T-cell interaction (22, 27, 49, 54, 65). Irrespective of the presence of viral antigens in infected APCs, the immune response eventually fails to control HIV-1 disease progression. An enigma exists, therefore, in the ability of DC to induce antiviral immunity while simultaneously facilitating virus propagation. While HIV-1-induced mutations leading to cytotoxic-T-lymphocyte (CTL) escape are thought to be the major cause of immune failure, recent studies have shown that the accumulation of immature dendritic cells in conjunction with the impaired processing and presentation of CTL epitopes could indeed be part of HIV-1 progression (41, 54, 63). Recent studies have also demonstrated that DC infected with HIV-1 selectively fail to mature, lack the potential to elicit mixed lymphocyte reactions, and are defective in interleukin 12 (IL-12) production (21, 46).

HIV-1 expresses a number of viral proteins, including the structural and accessory proteins that have been shown to dysregulate the host cellular immune response as part of viral immune evasive strategy. Exposure of DC to gp120 led to an upregulation of activation markers indicative of functional maturation. Despite their phenotype, however, these cells retained antigen uptake capacity and showed an impaired ability to secrete cytokines and chemokines and to induce T-cell pro-

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liferation (18). HIV-1 Tat has been shown to inhibit antigen-induced lymphocyte proliferation, while native Tat induces DC maturation (17, 60). Nef downregulates CD4 and major histocompatibility complex (MHC) class I molecules in T lymphocytes in order to escape the CTLs (13, 42). Nef has also been found to equip dendritic cells to inhibit alloreactive $CD8^+$ T-cell priming and triggers their apoptosis by upregulating tumor necrosis factor alpha and Fas L production by DC (42). HIV-1 Nef-induced upregulation of DC-specific ICAM-3 grabbing nonintegrin in dendritic cells facilitates lymphocyte clustering and viral spread (47). Nef induces chemokines in primary macrophages that are thought to facilitate lymphocyte recruitment and activation (47, 52). Though Tat and Nef are expressed early during infection, they are not packaged in the virus particle. HIV-1 Vpr, a 14-kDa accessory protein, is present in detectable levels in the virion, thus making it one of the first HIV proteins seen by the host cell (11). Vpr is necessary for the efficient infection of nondividing cells, such as macrophages, and enhances viral replication within T-cell lines and activated peripheral blood lymphocytes (8). Disease progression and infection in vivo is attenuated in patients with Vpr defects at the C terminus, indicating the importance of the role Vpr plays in viral pathogenesis (61).

HIV-1 Vpr is a pleiotropic protein that is known to dysregulate a number of host cellular events (cell cycle, apoptosis, and host gene expression) upon expression (8, 56, 66). Vpr is known to persist in different forms in vivo (free protein and a virion-associated form), thus exerting its effect on proximal and distal cells and tissues that are not infected by the virus (56). One particularly intriguing function of Vpr is its ability to mimic the immune suppressor glucocorticoids through its interaction with the glucocorticoid receptor (GR) and its response element, GRE (3, 29). We and others have recently shown that the presence of Vpr inhibits the induction of the immune response to the codelivered antigen in an in vivo model (4, 39). However, the effect of Vpr on dendritic cells and their function as APCs remain unexplored. In the present study, using an in vitro monocyte-derived DC and virus infection model, we address the role of Vpr in the regulation of dendritic cell maturation and T-cell activation and its potential implication in HIV-1 immune escape. We show that Vpr selectively impairs the expression of costimulatory molecules and maturation markers at both the protein and RNA levels. Moreover, the inhibition of IL-12p70 and upregulation of IL-10 by HIV-1 $vpr^+$-infected DC following lipopolysaccharide (LPS) or CD40 ligand (CD40L) stimulation was also observed. The ability of HIV-1 $vpr^+$ virus-infected DC to impair antigen presentation and activation of antigen-specific CTL clones demonstrates the relevance of Vpr in HIV-1 immunopathogenesis. Together, these results might in part explain the failure of the immune response to recall antigens and neoantigens that is observed in HIV-1 patients (53, 58).

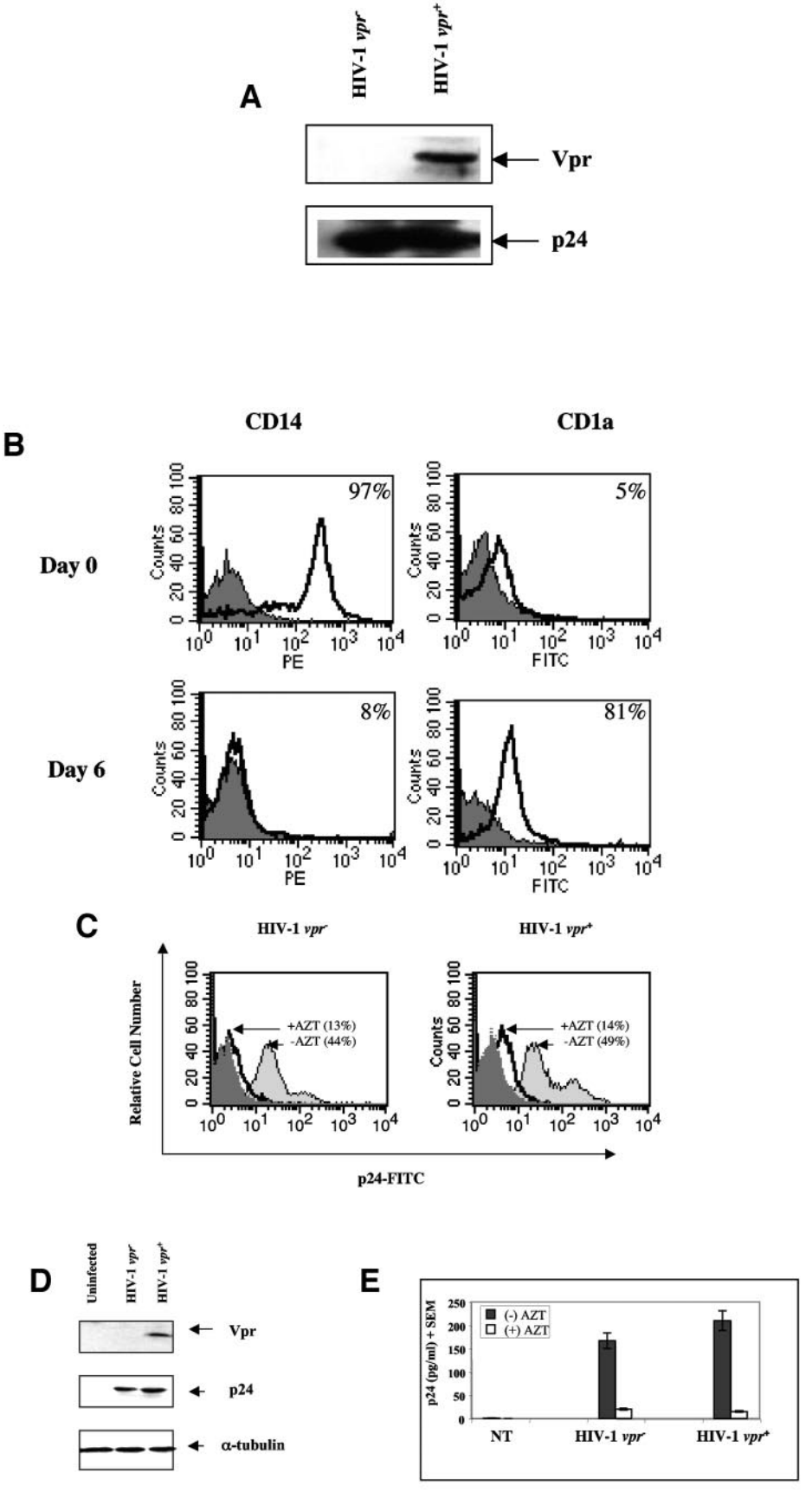
## MATERIALS AND METHODS

**Cell culture.** Monocyte-derived DC were generated from peripheral blood mononuclear cells (PBMCs). Heparinized blood samples were obtained with written consent from healthy donors. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. $CD14^+$ monocytes were purified by positive selection using anti-CD14 monoclonal antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as described previously (16). Briefly, PBMCs were resuspended in MACS buffer (2 mM EDTA, 0.05% bovine serum albumin in phosphate-buffered saline [PBS]) and incubated with anti-CD14 MACS beads at 4°C for 15 min. The cells were washed with MACS buffer and resuspended in MACS buffer before passage through a magnetic column. The purity of $CD14^+$ cells was tested by flow cytometry using CD14-phycoerythrin (PE) (BD-Pharmingen, San Diego, CA) and CD1a-fluorescein isothiocyanate (FITC) (ImmunoTech, Miami, FL) antibodies, and we observed that >95% of isolated cells were CD14 positive (Fig. 1B). To obtain monocyte-derived DC, $CD14^+$ cells ($0.5 \times 10^6$ cells/ml) were cultured in 60-mm culture plates in a total volume of 10 ml AIM-V (GIBCO, Carlsbad, CA) medium containing 25 ng/ml IL-4 (R&D Systems, Minneapolis, MN) and 50 ng/ml granulocyte-macrophage colony-stimuating factor (GM-CSF) (R&D Systems). Half the volume of medium was replaced every other day throughout the entire culture period as described previously (16). The plasmocytoma cell line J558, stably expressing surface CD40L (generated by Peter Lane, Switzerland), was maintained in Dulbecco's modified Eagle's medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Cambrex). Human HLA-A2-positive CD8 T-cell lines specific for melanoma antigen (gp100) and tyrosinase were a kind gift of Walter Storkus (University of Pittsburgh) and June Kan-Mitchell (Wayne State University), respectively, and were maintained as previously described (20). HEK 293T cells were obtained from Michelle Calos, Stanford University, and maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 1% L-glutamine (Cambrex), and 1% penicillin-streptomycin (GIBCO).

**Virus preparation and DC infection.** HIV-1 pNL43 $vpr^+$ and $vpr^-$ were pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) envelope and are described as HIV-1 $vpr^+$ and HIV-1 $vpr^-$, respectively. HEK293T cells ($2 \times 10^6$ per plate) were cotransfected with 7.5 μg of HIV-1 proviral construct (pNL43 $env^- vpr^+$ or pNL43 $env^- vpr^-$) and 2.5 μg VSV-G Env expression plasmid by the calcium phosphate precipitation method (6). Forty-eight hours posttransfection, the supernatants were collected, filtered through a 0.4-μm filter to remove cellular debris, and ultracentrifuged at 22,000 rpm for 1 h. The virus pellets were dissolved in PBS and stored at −80°C for subsequent assays. Virus titers were measured by p24 enzyme-linked immunosorbent assay (ELISA), and viral infectivity was assessed by determination of the 50% tissue culture infective dose using the HIV-1 reporter cell line cMAGI (AIDS Research and Reference Reagent Program [RRRP], National Institutes of Health [NIH]). Four-day-old DC ($5 \times 10^6$) maintained as described above were infected with HIV-1 $vpr^+$ and HIV-1 $vpr^-$ at a multiplicity of infection (MOI) of 2. Seventy-two hours postinfection, the cells were stimulated with either LPS (1 μg/ml; Sigma, St. Louis, MO), soluble CD40L (sCD40L) (100 ng/ml; Alexis, San Diego, CA), or gamma-irradiated CD40L-expressing J558 cells. For J558-CD40L stimulation, gamma irradiation was carried out for 14 min at 5,000 rads, and cells were added at a 10:1 ratio with DC.

**Western blotting.** A total of $5 \times 10^6$ uninfected and HIV-1 $vpr^+$- or HIV-1 $vpr^-$-infected DC were washed twice with PBS and lysed in RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1.0 mM phenylmethylsulfonyl fluoride, 0.05% deoxycholate, 10% sodium dodecyl sulfate, aprotinin (0.07 trypsin inhibitor unit/ml), and the protease inhibitors leupeptin, chymostatin, and pepstatin (1 μg/ml; Sigma). Cell lysates were clarified by centrifugation, and total cell lysates (50 μg) were separated on a 12 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred, and immunoblotted with anti-HIV-1 p24 (AIDS RRRP, NIH), anti-HIV-1 Vpr (a kind gift from John Kappes, University of Alabama), or anti-α-tubulin (NeoMarkers, Fremont, CA) antibodies. The blots were developed using an ECL kit (Amersham Biosciences, Piscataway, NJ).

**Flow cytometry.** To confirm the purity and differentiation of monocyte-derived DC, we tested the phenotype of the cells during this period by flow cytometry. Cells were stained with CD14-PE and CD1a-FITC or with a corresponding fluorochrome-conjugated immunoglobulin G (IgG) isotype control at days 0 and 6. The maturation of DC was evaluated by surface staining for CD80, CD83, CD86, and HLA-DR by flow cytometry. DC (infected and stimulated as described above) were washed twice with cold PBS (pH 7.2) containing 10% FBS and incubated with anti-CD80-PE, anti-CD83-PE, anti-CD86-PE (Immunotech), or anti-HLA-DR–PE and a mouse IgG2a-PE control (Caltag, Burlingame, CA) for 1 h at 4°C. The cells were washed three times with fluorescence-activated cell sorter (FACS) buffer. For the detection of intracellular p24, fixation and permeabilization were carried out using the cytoFix-cytoPerm kit (BD-Bioscience, Mountainview, CA). After two washes in Perm-Wash buffer (BD-Bioscience), intracellular p24 staining was performed at room temperature for 30 min using 5 μl of anti-p24-FITC antibody (Coulter, Miami, FL; clone KC47) per $10^6$ cells, followed by two washes in Perm-Wash buffer. The cells were gated in PE and FITC channels to quantitate the expression of DC markers in directly infected and exposed but otherwise uninfected subpopulations and analyzed by flow


A
HIV-1 vpr-
HIV-1 vpr+
Vpr
p24
B
CD14
CD1a
Day 0
Day 6
97%
5%
8%
81%
Counts
PE
FITC
C
HIV-1 vpr-
HIV-1 vpr+
Relative Cell Number
+AZT (13%)
-AZT (44%)
+AZT (14%)
-AZT (49%)
p24-FITC
D
Uninfected
HIV-1 vpr-
HIV-1 vpr+
Vpr
p24
α-tubulin
E
p24 (pg/ml) + SEM
(-) AZT
(+) AZT
NT
HIV-1 vpr-
HIV-1 vpr+

cytometry. Samples were analyzed using Epics-XL (Beckman Coulter, Miami, FL) with 5,000 gated events acquired for each sample, and the mean fluorescence intensity (MFI) was calculated using Cell Quest software (BD Biosciences).

**Real-time RT-PCR analysis.** Real-time reverse transcription (RT)-PCR was used to assess the transcriptional regulation of dendritic cell surface molecules. DC ($5 \times 10^6$) were cultured and infected with HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$ as described previously. Following 4 h of stimulation with LPS (1 μg/ml), the cells were collected by centrifugation and washed once with cold PBS, and total cellular RNA was extracted using the RNeasy minikit (QIAGEN, Valencia, CA) according to the manufacturer's protocol, with additional on-column DNase 1 digestion (RNase-free DNase kit, QIAGEN). The RNA concentration was determined by spectrophotometry, and the integrity was assessed by the 260/280 λ ratio and agarose gel electrophoresis. Two-step RT-PCR was performed as follows. RNA (0.2 to 0.5 μg) was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Real-time PCR was carried out in triplicate using commercially available primer-probe sets specific for CD80, CD83, CD86, and the ribosomal large protein (RPLPO). The comparative threshold cycle ($C_T$) method was used to determine the relative transcript ratio between control and treated samples. RNA levels were normalized to the RPLPO and calibrated to the uninfected LPS-stimulated sample. Internal controls consisting of untreated DC and LPS treatment alone were included to validate DC stimulation by costimulatory molecule transcription.

**FITC-dextran endocytosis.** DC (infected and uninfected) were stimulated with sCD40L LPS at a concentration of 1 μg/ml for 24 h, following which old media were replaced with fresh media. The cells were then incubated with FITC-dextran (molecular weight, 40,000; Sigma-Aldrich) at a concentration of 1 mg/ml for 50 min at either 37°C or 4°C. At the end of incubation, the cells were washed immediately with cold PBS, followed by cold FACS buffer. FITC-dextran was determined by flow cytometry as described above (44).

**Apoptosis assay.** Analysis of apoptosis was carried out using the Apoptosis Detection kit (BD Biosciences, San Diego, CA) in accordance with the manufacturer's instructions. Briefly, DC infected with HIV-1 *vpr*$^+$ and HIV-1 *vpr*$^-$ and stimulated for 24 h were washed twice with cold PBS and resuspended in sterile binding buffer containing 10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. The cells were incubated with annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark and diluted four times with binding buffer before being analyzed by flow cytometry. Uninfected and unstained cells were used to gate the DC on the forward and side scatter dot blot to estimate the percentage of PI- and annexin V-FITC-positive cells. To further confirm apoptosis biochemically, DC were lysed and immunoblotted using antibodies specific for full-length and cleaved caspase 3 (Cell Signaling Technology, Beverly, MA).

**Measurement of soluble cytokines by ELISA.** DC infected with HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$ were further stimulated with CD40L or LPS for 24 h. Following stimulation, the supernatants were collected and analyzed for the presence of cytokines. IL-12 p70 was measured by using Opti-EIA enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. Human IL-1β, IL-6, and IL-10 were measured using the Multicytokine Bead kit (Upstate, Lake Placid, NY) in a Luminex 100 instrument (Luminex, Austin, TX).

**IFN-γ ELISPOT assay.** To measure the CD8$^+$ T-cell-specific response to recall antigens, DC derived from HLA-A2-positive donors were infected with HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$, stimulated with CD40L cells as described above, and used in a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. Briefly, uninfected or infected DC stimulated as described above were loaded with melanoma peptide $gp100_{209–217}$ (ITDQVPFSV), $tyrosinase_{368–376}$ (YMNGTMSQV), EBV $BMLF1_{280–288}$ (GLCTLVAML), HIV-1 $p24_{19–27}$ (TL NAWVKGV), or influenza A virus $M1_{58–66}$ (GILGFVFTL) at 10 μg/ml and pulsed for 2 h at 37°C. Following the pulsing, the DC were washed twice with PBS and cultured with either CD8 T-cell lines (gp100, tyrosinase, and HIV-1 Gag) or autologous T lymphocytes (CD14$^-$ cells derived from the same DC donor; Epstein-Barr virus [EBV] and influenza virus) for 18 h. Antigen-specific T-cell responses were measured by IFN-γ ELISPOT as described previously (20, 24). Briefly, DC and T cells (ratios, 1:5 and 1:10) were added to 96-well nitrocellulose hemagglutinin plates (Millipore, Bedford, MA) that were precoated with human anti-IFN-γ monoclonal antibody 1DIK (Mabtech, Stockholm, Sweden) at a final concentration of 10 μg/ml for 18 h. Unpulsed DC with and without T cells, DC pulsed with unrelated HIV-2 Gag peptide with T cells, and T cells stimulated with 1 μg/ml phytohemagglutinin were used as controls. The cells were further incubated for 18 h and washed six times with PBS containing 0.05% Tween 20. To each well, biotinylated antibody specific for human IFN-γ (monoclonal antibody 7B6-1; Mabtech) was added at a concentration of 2 μg/ml, and the plates were incubated at 37°C for 2 hours. Following six washes in PBS containing 0.05% Tween 20, 100 μl of avidin-peroxidase complex was added to each well, and the plates were incubated at room temperature for 1 hour, washed, and developed with a solution containing 25 μl 30% $H_2O_2$ in 50 ml solution of dimethylformamide and 50 mM acetate buffer for 5 min. The reactions were stopped by washing the plates with running tap water, and the plates were dried. To count the spots, the plates were scanned using an automated ELISPOT reader. Each spot represented one IFN-γ spot-forming cell (SFC).

**Statistical analysis.** The results were expressed as mean ± standard error of the mean. The data were analyzed using the Student *t* test for normally distributed data with equal variances, and a *P* value of $<0.02$ was considered significant.

## RESULTS

**Production, uptake, and release of HIV-1 *vpr*$^+$ and HIV-1 *vpr*$^-$ in DC.** To evaluate the role of HIV-1 *vpr* in the context of infected DC, a single-cycle HIV-1 infection assay was performed as described previously (24). Envelope-defective proviral plasmids pNL43 *vpr*$^+$ and pNL43 *vpr*$^-$ were pseudotyped by cotransfection with VSV-G Env expression plasmid into HEK 293T cells. Forty-eight hours posttransfection, the culture supernatants were collected, and the viral titer was measured by p24 ELISA. The two proviral clones produced comparable amounts of virus (2.5 μg/ml). Virus pellets derived from 1 ml of culture supernatant were further characterized for the presence of Vpr and p24 (input virus for DC infection) by Western blotting. Comparable expression levels of p24 protein were found in HIV-1 *vpr*$^-$ and HIV-1 *vpr*$^+$ pellets. The deletion of *vpr* in the HIV-1 *vpr*$^-$ proviral DNA was confirmed by not detecting the 14-kDa Vpr band in the HIV-1 *vpr*$^-$ virus pellet but detecting it in the HIV-1 *vpr*$^+$ pellet (Fig. 1A).

Next, we characterized the DC to confirm the differentiation process, as well as the purity, prior to infection and activation by CD40L or LPS (Fig. 1B). Our results demonstrate that at day 0, about 97% of the population was CD14 positive and

FIG. 1. Virus preparation, infection, and expression of viral proteins in infected DC. (A) VSV-G Env-pseudotyped HIV-1 *vpr*$^+$ and HIV-1 *vpr*$^-$ were prepared as described in Materials and Methods and further characterized for the presence of Vpr by immunoblot analysis using p24- and Vpr-specific antibodies. (B) PBMCs were isolated from healthy donors. CD14$^+$ monocytes were purified by positive selection using anti-CD14 monoclonal antibody-coated magnetic microbeads. The purity of the cells isolated for myeloid-derived monocytes was tested by flow cytometry using CD14- and CD1a-specific antibodies. Expression of CD14 and CD1a on day 0 (top) and day 6 (bottom) of culture in the presence of IL-4 and GM-CSF. The dark-gray histogram represents the corresponding IgG control. The white histogram represents expression of CD14- and CD1a-positive cells. This experiment was repeated several times. (C) PBMC-derived CD14$^+$ monocytes were isolated and cultured with GM-CSF and IL-4 to generate DC. On day 4, the cells were infected with HIV-1 *vpr*$^+$ and HIV-1 *vpr*$^-$ at an MOI of 2.0 and incubated further in DC culture medium in the presence (+) and absence (−) of AZT (1 μM) with an IgG control. Three days postinfection, the cells were analyzed by flow cytometry to identify the number of infected cells using p24-FITC antibody and FACS analysis. DC were cultured in the presence (white histogram) and absence (light-gray histogram) of AZT (1 μM) with an IgG control (dark gray histogram). (D) Infected DC were lysed and immunoblotted to detect the presence of the viral proteins Gag (p24) and Vpr; α-tubulin was used as an internal loading control. (E) Quantitation of virus particles released into the culture medium by infected DC in the presence and absence of AZT was done by p24 ELISA. Each experiment was repeated at least six times, and similar results were obtained.

only 5% was CD1a positive, indicating a phenotype typical of myeloid-derived monocytes. In contrast, at day 6 of culture a majority (81%) of the cells were found to express CD1a marker on their surfaces, and only 8% of the cells were CD14 positive. These data represent a phenotypic profile characteristic of truly differentiated immature DC.

Immature DC ($0.5 \times 10^6$ cells/ml) at day 4 of culture were infected with equal amounts (MOI, 2) of HIV-1 *vpr*$^-$ or HIV-1 *vpr*$^+$ for 24 h, and the cells were washed and maintained for another 2 days in medium before stimulation. To determine the percentage of cells infected with HIV-1 *vpr*$^-$ and HIV-1 *vpr*$^+$, the cells were stained 3 days postinfection, fixed, and analyzed by flow cytometry for p24 antigen using anti-p24 antibody (Fig. 1C). Uninfected DC demonstrated low levels of background staining (~10%). Compared to uninfected cells, 44% of total DC were found to be positive for HIV-1 *vpr*$^-$ infection, whereas 49% of DC were positive for HIV-1 *vpr*$^+$. Additionally, our immunofluorescence studies to detect viral antigens in DC also support a similar percentage (45 to 60%) of infection in DC with these two viruses (data not shown). Next, to confirm that infected DC expressed comparable levels of viral proteins (Gag and Vpr), we performed a Western blot analysis. Three days postinfection, DC were lysed and whole-cell lysates were probed with anti-p24 and anti-Vpr antibodies (Fig. 1D). The results indicated that DC infected with VSV-complemented HIV-1 *vpr*$^-$ and HIV-1 *vpr*$^+$ expressed the respective viral proteins encoded by the proviral genome. Additionally, virus particles released into the culture supernatants by DC infected with HIV-1 *vpr*$^-$ and HIV-1 *vpr*$^+$ were quantitated by p24 ELISA using the standard p24 kit (Fig. 1E). Cells infected with HIV-1 *vpr*$^+$ showed slightly increased virus production based on both extra- and intracellular p24 expression (p24 ELISA and Western blotting) compared to that detected in HIV-1 *vpr*$^-$-infected cells. The results indicated that HIV-1 *vpr*$^+$-infected DC showed expression of p24 and Vpr, whereas HIV-1 *vpr*$^-$-infected cells showed only the presence of p24. Furthermore, expression of viral antigens in infected DC was inhibited by pretreating the cells with zidovudine (AZT; 1 μM) and analyzed by FACS and p24 released into the medium (Fig. 1C and E). These results further indicate that DC support moderate-level HIV-1 infection through the long terminal repeat promoter. We also detected the presence of p24 in AZT-treated cells (about 14%) in both the *vpr*$^-$ and *vpr*$^+$ virus-infected DC populations, indicating that this could be due to the virus particles endocytosed by DC rather than the de novo synthesis of Gag.

**HIV-1 Vpr impairs phenotypic maturation of DC promoted by LPS or CD40L.** The upregulation of costimulatory molecules (CD80 and CD86) and maturation marker (CD83) is critically required for the induction of an effective DC-mediated adaptive immune response (5). To determine the effects of HIV-1 Vpr on the modulation of these surface molecules in vitro and its functional impact on host immune responses, DC infected with HIV-1 *vpr*$^-$ and HIV-1 *vpr*$^+$ were further stimulated with LPS or irradiated J558 CD40L cells (ratio of DC to CD40L cells = 1:10) for 24 h. Immunophenotyping of these cells was performed to analyze the expression of different costimulatory molecules and the maturation marker. Initial analysis of the total HIV-1-infected DC population (following CD40L stimulation) indicated that expression (measured as MFI) of CD80 and CD86 was found to be impaired in cells infected with HIV-1 *vpr*$^+$ compared to those infected with HIV-1 *vpr*$^-$. In order to investigate if the observed Vpr effect was confined to the infected DC population or generally distributed in the entire population, cells were double stained for p24 and the surface markers CD80, CD83, CD86, and HLA-DR or IgG isotypes. The data presented in Fig. 2 indicate that there was a reduction of 60 to 70% (measured as MFI) in CD80 and CD86, whereas CD83 was reduced by 30% in the HIV-1 *vpr*$^+$-infected p24-positive DC population following CD40L stimulation compared to HIV-1 *vpr*$^-$-infected p24-positive DC. In addition, we noted a slight reduction (30%) in the expression of CD80, CD83, and CD86 in the uninfected (p24-negative population) DC infected with HIV-1 *vpr*$^+$ compared to DC infected with HIV-1 *vpr*$^-$. We propose that this effect could be due to the exposure of DC to noninfectious virus particles containing Vpr as a virion-associated molecule. No significant downregulation of HLA-DR was observed among *vpr*$^-$ and *vpr*$^+$ virus-positive activated DC.

Similarly, about 60% reduction in MFI for CD80 and 30% for CD86 in HIV-1 *vpr*$^+$-infected DC populations was noticed compared to HIV-1 *vpr*$^-$-infected cells when stimulated with LPS. HIV-1 *vpr*$^+$ treatment resulted in an eightfold decrease in MFI and a 40% reduction in total CD83-positive cells upon LPS stimulation in comparison to HIV-1 *vpr*$^-$ treatment (data not shown). It is interesting that Vpr did not alter the percentages of CD80- and CD86-positive cell populations, suggesting that Vpr did not eliminate the cells by apoptosis but rather inhibited the expression levels of surface molecules. The selective nature of Vpr-induced impairment of DC surface markers was further demonstrated when the expression of HLA-DR was found to be unaltered in HIV-1 *vpr*$^+$- and HIV-1 *vpr*$^-$-infected DC upon CD40L (Fig. 2) or LPS stimulation. No measurable difference was observed in the expression of these DC surface markers in HIV-1 *vpr*$^+$- and HIV-1 *vpr*$^-$-infected cells in the absence of a second signal (data not shown), suggesting that the observed Vpr-mediated down-modulation is linked to activation-induced maturation of DC. Taken together, these data indicate that both synthesized (de novo) Vpr from infected cells and virion-associated Vpr could selectively induce the downregulation of DC phenotypic markers that are important for T-cell activation.

**Vpr-induced dysregulation of DC phenotypic maturation is independent of apoptosis.** Treatment with recombinant Vpr protein or endogenous expression of HIV-1 Vpr has been shown to induce apoptosis in different cell types in a dose-dependent manner (51, 66). In order to assess whether this profound effect of Vpr on DC phenotypic maturation is due to apoptosis, *vpr*$^-$ or *vpr*$^+$ virus-infected DC with or without LPS or CD40L were labeled with annexin V-FITC and -PI and analyzed by flow cytometry. Figure 3 represents the DC infected at an MOI of 2 with HIV-1 *vpr*$^-$ or HIV-1 *vpr*$^+$, which is the dose used in all our experiments. Our results showed that only a small percentage of cells (10 to 12%) underwent apoptosis in both HIV-1 *vpr*$^-$-infected and HIV-1 *vpr*$^+$-infected DC populations. Uninfected DC showed similar induction of apoptosis, whereas in UV-treated cells used as a positive control, about 98% of the cells were apoptotic. In each condition, these numbers include about 6% of the cells that were both PI and annexin V positive, thereby representing either a late

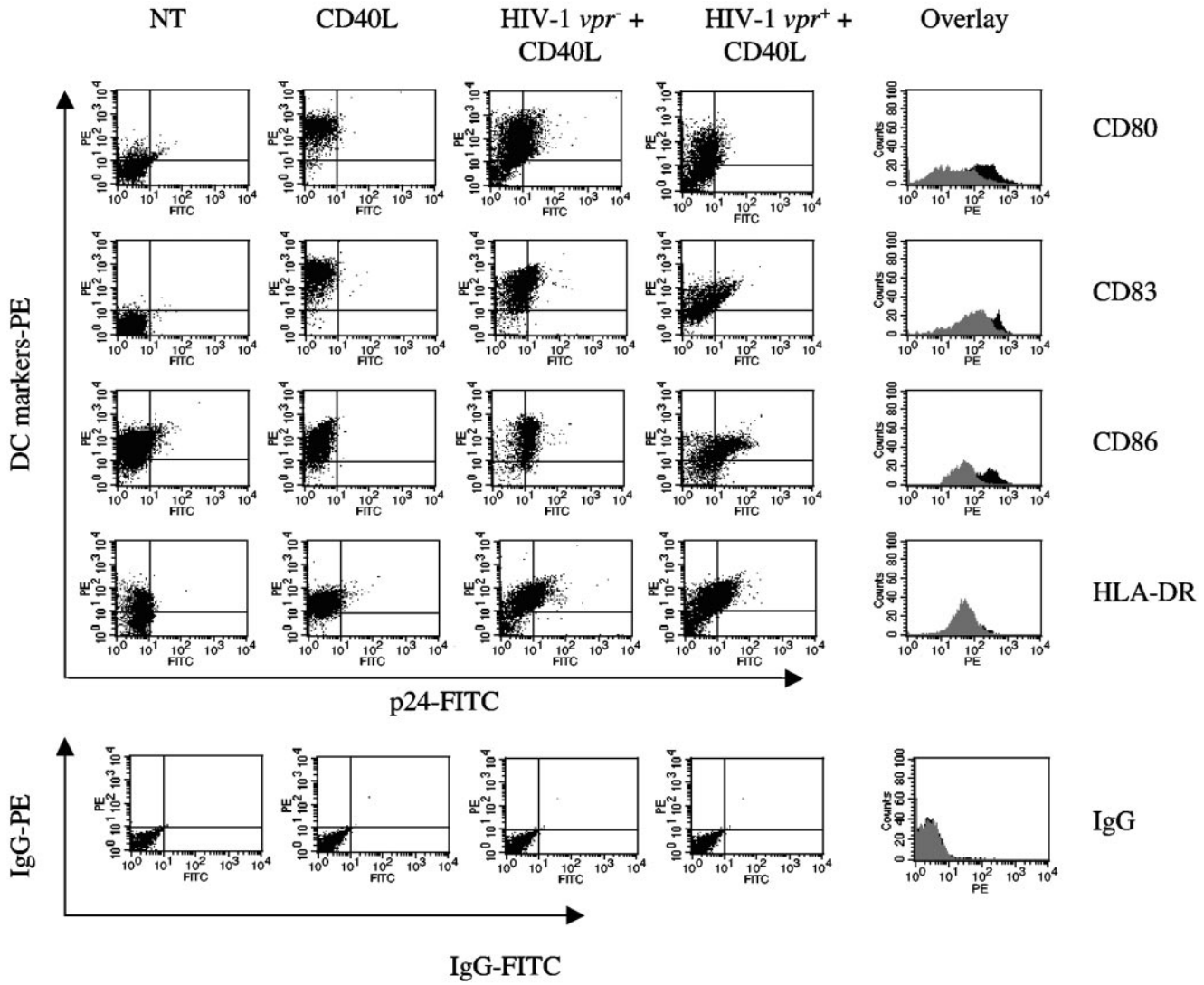



FIG. 2. Immunophenotyping of DC infected with HIV-1 $vpr^+$ and HIV-1 $vpr^-$. Immature DC were infected with HIV-1 $vpr^+$ or HIV-1 $vpr^-$ as described in Materials and Methods, stimulated with either LPS or cells expressing CD40L, and subsequently analyzed for phenotype by direct flow cytometry. Infected DC were stimulated with irradiated CD40L-expressing J558 cells and assessed for CD80, CD83, and CD86 cell surface molecules using directly conjugated specific antibodies and isotype controls followed by intracellular p24 staining with IgG control, as described earlier. Viable DC were gated on forward and side scatter dot blots and analyzed for surface expression of DC markers in p24-negative uninfected (left upper quadrant) or p24-positive infected (right upper quadrant) populations. The bottom row represents corresponding IgG controls. The overlay column represents the histograms for CD40L-stimulated DC infected with HIV-1 $vpr^-$ (black histogram) and CD40L-stimulated DC infected with HIV-1 $vpr^+$ (dark gray histogram). NT, no treatment. The data are representative of four similar experiments.

apoptotic or necrotic population, consistent with our trypan blue assay for cell viability (data not shown). Additionally, CD40L or LPS stimulation did not change the overall percentage of cells undergoing apoptosis in any of these cultures. However, cells infected with higher doses of HIV-1 $vpr^+$ (MOI, more than 5 to 8) induced a higher level of apoptosis in these cells (data not shown). This result supports the idea that the dose of HIV-1 $vpr^+$ at an MOI of 2 used in this study could modulate DC phenotypic maturation without inducing apoptosis.

Caspases are routinely used as a measure of apoptosis, in contrast to necrosis. Caspase 3 activation occurs at the intersection of all caspase-dependent pathways and therefore is an excellent marker of caspase-dependent apoptotic death. We sought to identify whether caspase 3 is activated during DC infection to further confirm our cytometry data. Immunoblot analysis confirmed the expression of inactive or full-length caspase 3 in HIV-1 $vpr^-$- and HIV-1 $vpr^+$-treated DC without any detectable expression of cleaved caspase 3 (Fig. 3B), indicating that the DC underwent activation without any marked activation of apoptosis. This is in agreement with previous studies showing that HIV-1 infection itself did not induce a significant level of apoptosis in the infected DC (24).

**Vpr-mediated downregulation of costimulatory molecules occurs at the mRNA level.** Potential explanations for the decrease in cell surface expression of CD80, CD86, and CD83

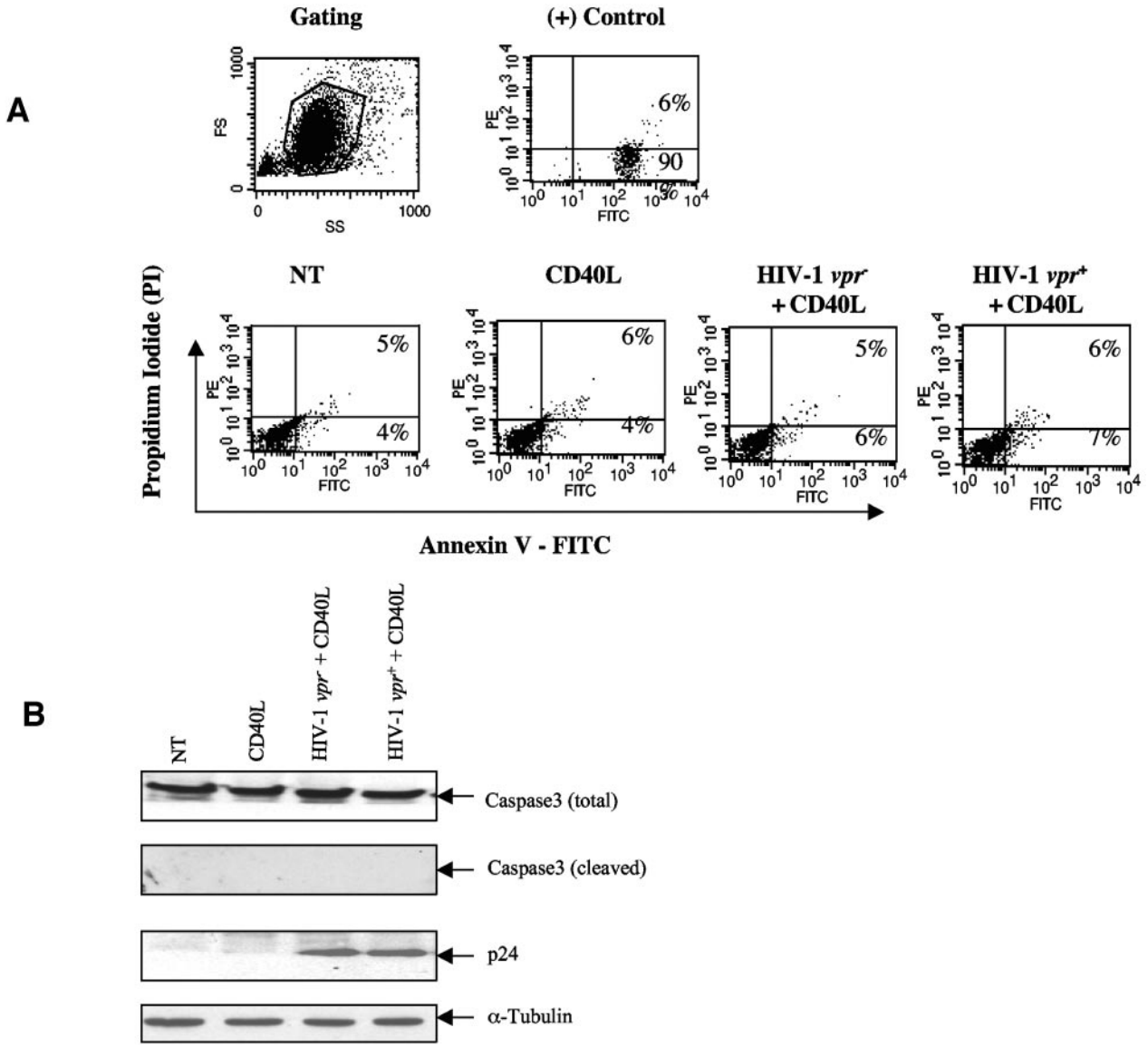



FIG. 3. Induction of apoptosis by CD40L- or LPS-stimulated DC infected with HIV-1 $vpr^-$ or $vpr^+$. DC were infected and stimulated as described in Materials and Methods. Induction of apoptosis following infection and stimulation was detected by staining the DC with PI and annexin V-FITC antibodies. (Top left) Gating of DC on forward and side scatter dot blots. (Top right) UV-irradiated DC as a control. (Bottom) Apoptotic DC populations with no treatment (NT), CD40L-stimulated uninfected, and HIV-1 $vpr^-$- and $vpr^+$-infected and stimulated DC. The number in the upper right quadrant indicates the percent necrotic cells (PI and annexin V positive). The lower right quadrant in each blot indicates the apoptotic population (annexin V-positive cells). (B) Expression of total and cleaved caspase 3 in DC by immunoblot analysis. The results are representative of three similar experiments.

seen by flow cytometry may be either increased internalization and degradation of these proteins or changes in the transcriptional level. We utilized real-time RT-PCR to investigate whether the observed changes in the protein levels of these costimulatory molecules were correlated with changes in their mRNA levels. As described in Materials and Methods, DC were infected with HIV $vpr^-$ or HIV $vpr^+$ and stimulated with LPS (1 μg/ml) for 4 hours. Following stimulation, the cells were collected and lysed, RNA was isolated, and the levels of CD80, CD86, and CD83 were assessed. Real-time RT-PCR was carried out using the comparative $C_T$ method to generate relative ratios of gene expression between samples (Table 1). In a detailed study of real-time RT-PCR endogenous controls by Lossos et al. (34), RPLPO was determined to show the least variance of the tested endogenous controls in unstimulated versus stimulated human primary T cells. We found that equivalent amounts of RNA yielded almost identical RPLPO expression, validating the use of this gene as an endogenous control in our dendritic cell system (Fig. 4D). Samples were first calibrated to the LPS-stimulated cells, setting this sample at 1.0 and determining relative ratios. This calculation, presented in Table 1 as the ratios relative to LPS treatment,

TABLE 1. Real-time RT-PCR analysis of DC costimulatory molecules

| Treatment | Ratio relative to LPS treatment (95% CI)[a] | | |
|---|---|---|---|
| | CD80 | CD83 | CD86 |
| LPS | 1.0 (0.94–1.06) | 1.0 (0.93–1.08) | 1.0 (0.89–1.12) |
| HIV-1 *vpr*$^-$ + LPS | 1.57 (1.40–1.75) | 1.01 (0.87–1.17) | 1.06 (0.94–1.19) |
| HIV-1 *vpr*$^+$ + LPS | 0.23 (0.21–0.26)* | 0.24 (0.22–0.27)* | 0.27 (0.26–0.27)* |

[a] DC were infected with HIV-1 *vpr*$^-$ or HIV-1 *vpr*$^+$, followed by 4 hours of stimulation with LPS (1 μg/ml). RNA (0.33 μg) was reverse transcribed, and real-time RT- PCR was performed in triplicate using the appropriate Taqman primer-probe sets (Applied Biosystems, CA). Ratios were determined via the comparative $C_T$ method ($\Delta\Delta C_T$), using RPLPO to normalize and the indicated sample as the calibrator (LPS or HIV-1 *vpr*$^-$). Internal controls consisting of DC treated with LPS were included to validate DC stimulation by costimulatory molecule transcription. The data are representative of four independent experiments. Statistical significance was calculated for the ratio of HIV-1 *vpr*$^+$ to HIV-1 *vpr*$^-$; *, $P < 0.0001$. CI, confidence interval.

served as an internal control indicating successful stimulation. In order to determine the specific effects of Vpr, the HIV *vpr*$^-$ sample was used as a calibrator and the relative ratio of expression of each gene in the HIV *vpr*$^+$ samples was determined. Given that these viruses differ only in their expression of Vpr, this system allows the assessment of the effects of Vpr in a viral infection without the compounding effects of other HIV genes on transcription. Our results indicated that HIV-1 *vpr*$^+$ significantly downregulated the mRNA levels of CD80, CD86, and CD83 (Fig. 4 and Table 1). This effect cannot be attributed to Vpr-mediated global downregulation of transcription, as RPLPO levels were not changed upon HIV *vpr*$^+$ infection. Together, these findings demonstrate the ability of Vpr to selectively impair the transcriptional upregulation of immunomodulatory molecules upon signal-induced activation.

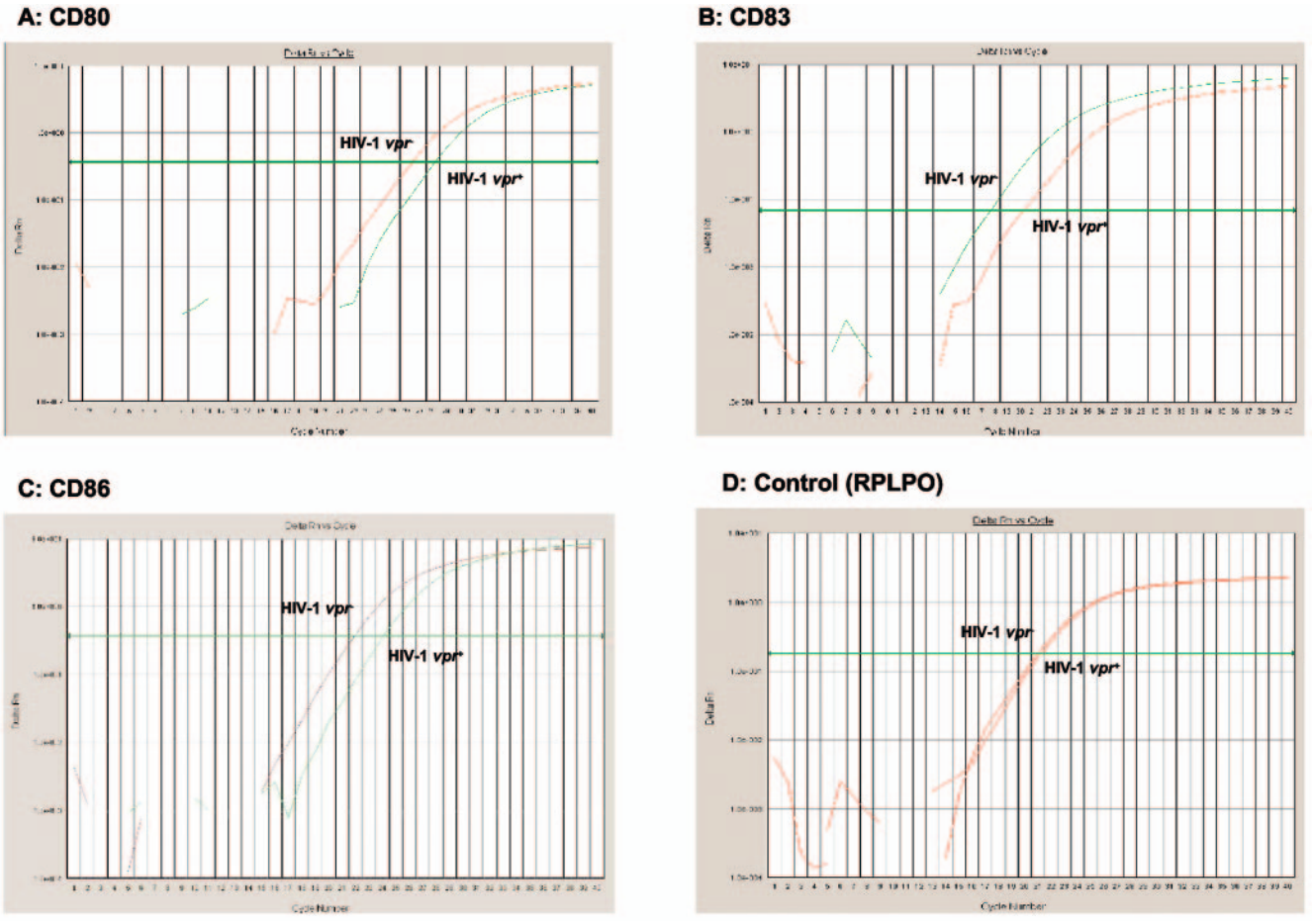



FIG. 4. Downregulation of DC costimulatory molecule mRNA expression by real-time RT-PCR. DC were cultured and infected as previously described, followed by 4 hours of incubation with LPS. Real-time RT-PCR was carried out on an ABI 7000 using primers and probes specific for CD80 (A), CD83 (B), CD86 (C), or the internal control RPLPO (D). The figure is representative of data attained from experiments performed in triplicate with three separate donors. The $C_T$ used to calculate the relative ratio was the cycle number ($x$ axis) at which probe-specific fluorescence crossed the threshold line (dark horizontal line) as set by ABI PRISM 7000 Sequence Detection System software. Colored lines are defined by adjacent labels.

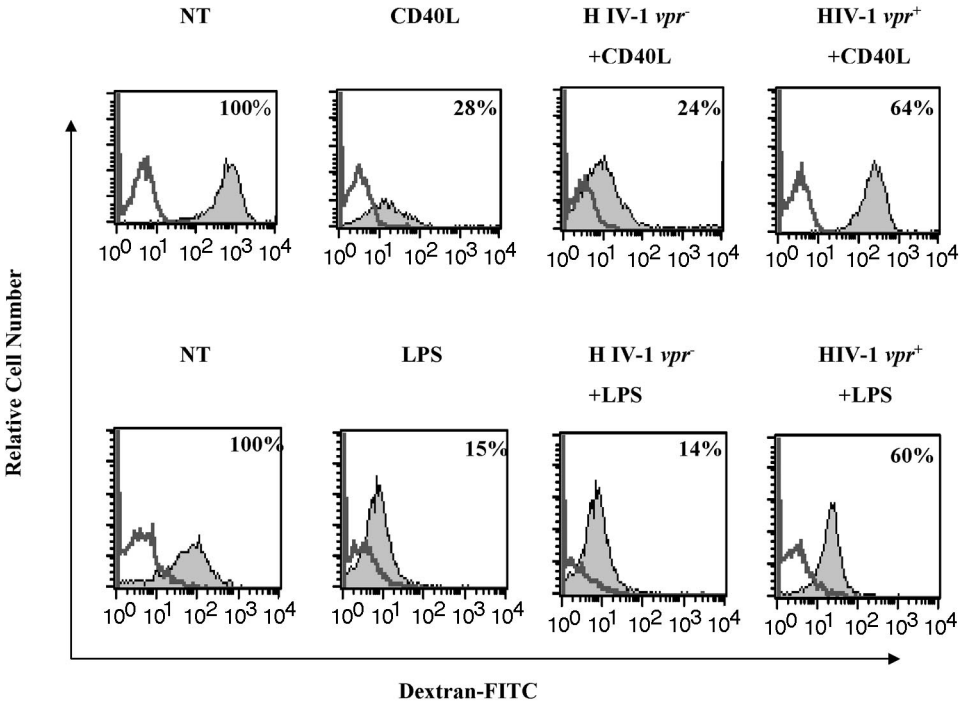



FIG. 5. Effect of HIV-1 Vpr on particle uptake by immature DC. Immature DC were infected with HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$ as described in Materials and Methods and cultured for 3 days. Following 24-h CD40L (top row) or LPS (bottom row) stimulation, the cells were incubated with FITC-dextran for 50 min at 37°C or 4°C, and antigen uptake was assessed by flow cytometry. The filled light-gray histograms represent antigen uptake at 37°C; the white histograms indicate antigen uptake at 4°C. The value inside each histogram is the percent endocytosis at 37°C. Antigen uptake by unstimulated and uninfected DC at 37°C was considered to be 100%, calculated based on the corresponding MFI. NT, no treatment. The data are representative of four similar experiments, each performed in triplicate.

**DC infected with HIV-1 *vpr*$^+$ display the functional phenotype of immature DC upon ligand-induced activation.** Given these potent effects of Vpr on dendritic cells in vitro, we next investigated whether the observed dysregulation of DC phenotypic maturation in response to Vpr was also mirrored in the endocytic function upon signal-induced activation. To address this, DC were infected with HIV-1 *vpr*$^+$ or HIV-1 *vpr*$^-$, stimulated with CD40L or LPS, and assessed for the ability to internalize soluble fluorescent antigen as a measure of functional maturation. DC were incubated with FITC-dextran at a concentration of 1 mg/ml for 50 min at 37°C. As a control, a second set was incubated at 4°C for the same period of time. Endocytosis was measured by flow cytometry using the FITC channel. The results are presented as percent endocytosis, where the unstimulated and uninfected DC are considered 100%. Figure 5 shows the endocytosis profile of HIV-1 *vpr*$^-$- and HIV-1 *vpr*$^+$-infected DC in the context of CD40L or LPS stimulation. DC stimulated with CD40L showed only 28% FITC-dextran-positive cells. In the case of DC infected with HIV-1 *vpr*$^-$ followed by CD40L stimulation, a 24% uptake similar to that with CD40L stimulation alone was observed. In sharp contrast, HIV-1 *vpr*$^+$-infected DC showed 64% FITC-dextran cells, suggesting that these cells exhibit an immature phenotype. DC incubated at 4°C showed less than 10% uptake in all experimental groups, which could be due to binding (Fig. 5, top row). It is interesting that Vpr-exposed DC, although destined to undergo a defective maturation cascade, maintain the functional characteristics of immature DC. A similar pattern was observed when we used LPS to trigger DC maturation (Fig. 5, bottom row). These data are compatible with those of flow cytometry and real-time RT-PCR analyses of surface molecules, suggesting that Vpr might selectively impair the phenotypic maturation of infected DC upon activation and that these cells retain the functions of immature DC.

**DC are deficient in proinflammatory cytokine production in the presence of Vpr.** It has been well established that stimulation of DC results in increased production of an array of proinflammatory cytokines, such as IL-12, IL-10, IL-6, and tumor necrosis factor alpha. These cytokines play crucial roles in the DC-mediated Th1-Th2 response and cross priming of cytotoxic T lymphocytes (7, 9, 10, 31, 43). To analyze whether HIV-1 *vpr*$^+$-exposed DC are deficient in cytokine production followed by microbial stimuli or CD40L, a Luminex-based cytokine bead array was performed with supernatants collected at 24 h following stimulation (Fig. 6). Following stimulation with CD40L, the uninfected DC produced 725.7 pg/ml IL-12 compared to the undetectable level ($<$5 pg/ml) observed in DC with no stimulation. HIV-1 *vpr*$^-$-infected DC produced a level (780.1 pg/ml) of IL-12 similar to that of CD40L-stimulated uninfected DC, whereas DC infected with HIV-1 *vpr*$^+$ upon CD40L stimulation produced $<$50% (383.0 pg/ml) of IL-12. It is interesting that HIV-1 *vpr*$^+$ infection markedly suppressed the amount of IL-12p70, but IL-6 production remained unchanged when stimulated by CD40L compared to that with *vpr*$^-$ virus (Fig. 6, top two rows). Similarly, altered production of proinflammatory cytokines was observed when HIV-1 *vpr*-

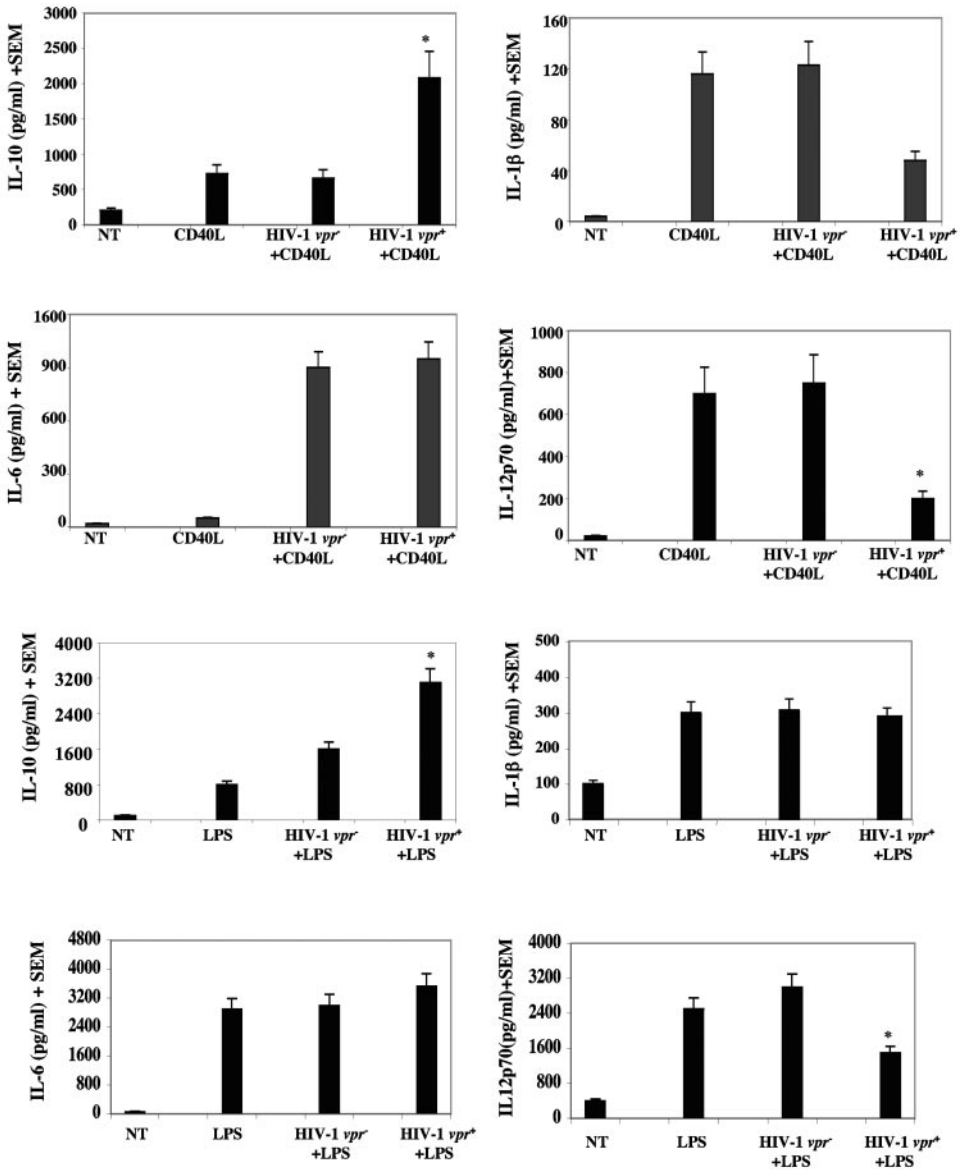



FIG. 6. Production of proinflammatory cytokines from infected DC, followed by CD40L-induced maturation. DC treated with HIV-1 $vpr^-$ or HIV-1 $vpr^+$ were stimulated with CD40L (top two rows) or LPS (bottom two rows) for 24 h. DC culture supernatants were collected and assayed for IL-12p70, IL-1β, IL-6, and IL-10 using a cytokine bead array kit in a Luminex 100. NT, no treatment. The results are representative of six independent experiments, each performed in triplicate. *, $P < 0.02$ compared to DC infected with HIV-1 $vpr^-$ and stimulated with CD40L or LPS.

infected DC were stimulated with LPS for 24 h (Fig. 6, bottom two rows). Interestingly, IL-10 was upregulated two- to threefold in the presence of Vpr compared to the HIV-1 $vpr^-$-infected DC upon stimulation with either CD40L or LPS (Fig. 6). These results, in conjunction with deficient expression of costimulatory molecules, indicate that HIV-1 $vpr^+$ infection might induce a shift in cytokine profile from Th1 to Th2 and polarize DC toward a nonimmunogenic phenotype as a result of tolerance.

**HIV-1 Vpr impairs the potency of CD40L-activated DC to elicit an antigen-specific T-cell response.** Maturation of DC is characterized by the upregulation of costimulatory molecules and their ability to efficiently prime and stimulate the antigen-specific T-cell response. Given the impaired effects of Vpr in terms of phenotypic and functional maturation of DC in vitro, we determined if Vpr-treated DC were capable of stimulating antigen-specific $CD8^+$ T cells and autologous memory T lymphocytes. DC generated from normal PBMCs with the HLA-A2-positive phenotype were infected with HIV-1 $vpr^-$ or HIV-1 $vpr^+$ and stimulated with CD40L for 24 h. For T-cell cytotoxic effector functions, CD8-derived cell lines specific for tyrosinase or gp100 peptide were used as responders. DC were pulsed for 2 hours with either tyrosinase or gp100 peptide, washed, and then coincubated with responder CD8 cells for 18 h. The $CD8^+$ T-cell response was then measured by IFN-γ ELISPOT assay. In the case of tyrosinase-specific CTL clones, uninfected or HIV-1 $vpr^-$-infected DC stimulated with CD40L induced a significant IFN-γ response (1,900 SFCs/$10^6$ cells).

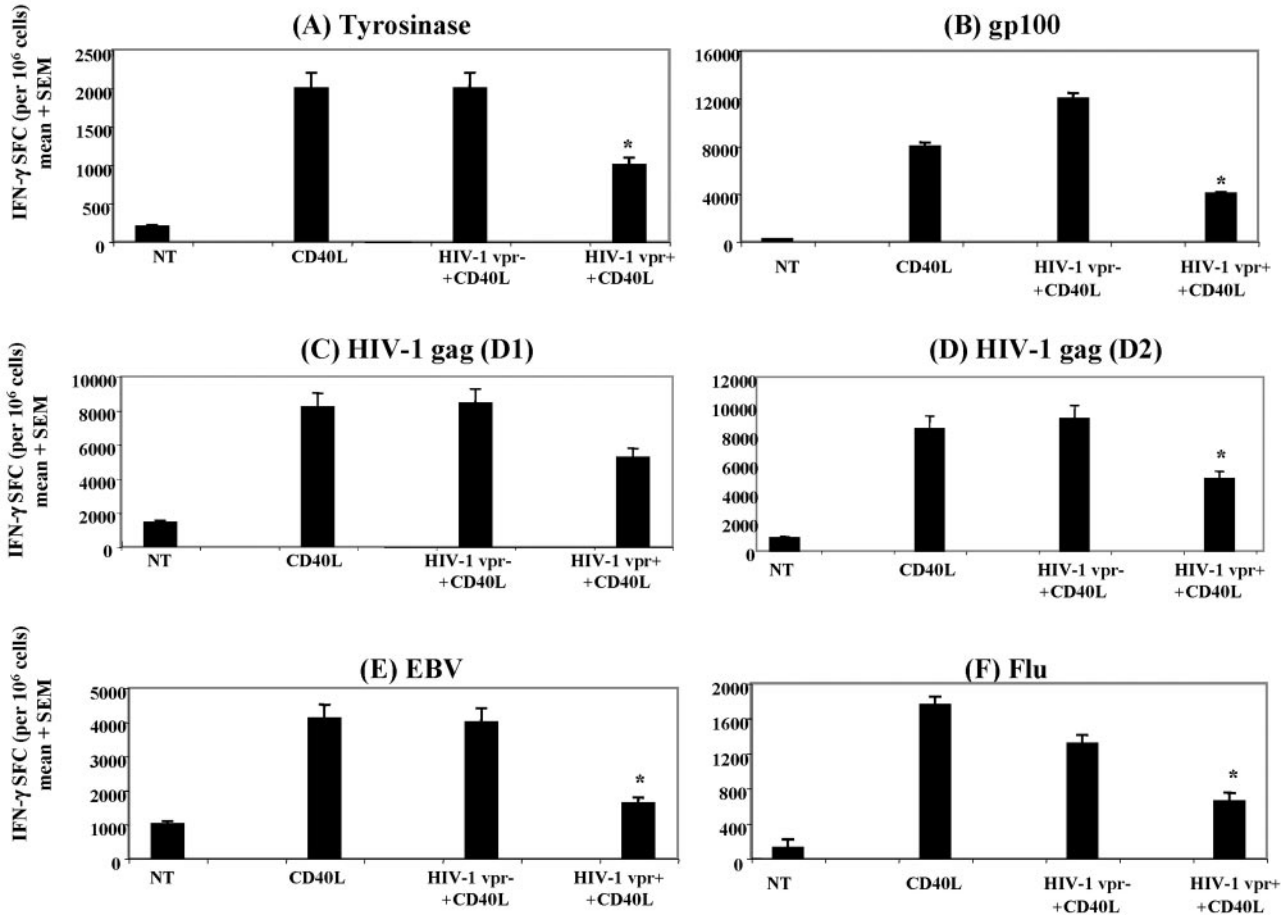



FIG. 7. Antigen presentation by virus-infected DC measured by IFN-γ ELISPOT. (A to D) HLA-A2-specific uninfected DC and DC infected with either HIV-1 $vpr^+$ or HIV-1 $vpr^-$ were loaded with different A2-specific cytotoxic-T-cell peptides (tyrosinase, gp100, and $p24^{gag}$) and subsequently coincubated with a $CD8^+$ peptide-specific T-cell line in an IFN-γ ELISPOT plate. Antigen-specific immune response was measured by the ability of the T cells to produce IFN-γ as determined by ELISPOT assay. The results are expressed as IFN-γ SFCs per $10^6$ cells. The data represent one out of two independent experiments performed in triplicate. (E and F) DC derived from an HLA-A*0201 donor with influenza virus (Flu) and chronic EBV infections were cultured and infected with $vpr^-$ or $vpr^+$ viruses and stimulated with CD40L. The cells were then pulsed with EBV or influenza virus for 2 hours before responder cells were added. Autologous CD14 T cells derived from the same EBV- and influenza virus-infected donor were added at ratios of 1:10 and 1:5 (DC:T cell) for 18 h. The antigen-specific T-cell response was measured by IFN-γ ELISPOT assay using specific antibodies. NT, no treatment. The data are representative of three similar experiments, each performed in triplicate. *, $P$ value $< 0.02$ compared to HIV-1 $vpr^-$-infected cells.

Considering this to be 100%, HIV-1 $vpr^+$-infected DC were found to elicit a <50% IFN-γ-specific T-cell response, indicating that DC generated in the presence of Vpr are defective in the ability to stimulate $CD8^+$ cells to specific peptide antigens (Fig. 7A). A similar response was observed with a $CD8^+$-specific cell line for gp100 (Fig. 7B). Together, these results further support the idea that Vpr-induced phenotypic dysregulation of DC reflects functional impairment and that the observed defect in T-cell priming is global rather than specific to a particular antigen. To further correlate the Vpr effect in the context of HIV-1 antigens, we tested two cell lines derived from different HLA-A2 donors that are specific for p24 Gag (TV9 peptide) using an ELISPOT assay (Fig. 7C and D). The results indicate that infection of DC with HIV-1 $vpr^+$ resulted in a functional defect, as observed in their impaired ability to elicit IFN-γ-specific T-cell response.

Next, to assess the Vpr-mediated effects on DC ability to recall antigens, we used DC derived from an HLA A*0201-positive donor who had an established CTL response for EBV and influenza virus antigens. DC isolated from this donor were similarly infected and activated and subsequently loaded with EBV or influenza virus peptides to stimulate autologous memory $CD14^-$ T cells. Similar HIV-1 Vpr-mediated impaired $CD8^+$ cell-specific IFN-γ responses were observed (Fig. 7E and F). These results indicate that Vpr initiates a negative regulatory signal through defective antigen presentation and T-cell priming, which equips the HIV-1 $vpr^+$-infected DC to disarm the adaptive immune response.

## DISCUSSION

Effective antiviral immunity against viral infection requires both innate and adaptive immune responses. Many viruses, including HIV-1, have evolved ways to target the immune cells,

DC, and T and B lymphocytes, the major players in inducing humoral and cellular responses, as part of their immune escape mechanisms (2, 12, 55). One of the pathways viruses effectively use is the impairment of antigen presentation and T-cell activation. DC are susceptible to HIV-1 infection in vivo, and these cells are one of the initial targets at the mucosal surface (22, 36, 48, 52). Eventually, the virus manages to survive in DC and to use the cells to facilitate virus dissemination without being detected by the host immune system. Here, we report one of the potential mechanisms that HIV-1 might effectively use to evade immune surveillance. Using an in vitro dendritic cell and virus infection model, we have shown that one of the virion-associated accessory proteins, Vpr, plays an important role in phenotypic and functional alterations of DC. This could very well explain the presence of immature DC exhibiting low expression of costimulatory molecules during acute and chronic HIV-1 infections in lymphoid tissues (33).

It is now well established that DC not only take up the virus particles but also support HIV-1 infection and express HIV-1 viral proteins through the long terminal repeat promoter (24). HIV-1 accessory gene products are known to regulate the host cellular immune response at multiple levels (13, 18, 60). In addition to the structural and enzymatic proteins that are necessary for virus binding and infection, HIV-1 virions also include the accessory gene product Vpr, suggesting that Vpr might play a role during early events (as a virion-associated molecule) and during productive viral infection (de novo expression). Despite Vpr's role in cell cycle arrest and apoptosis in proliferating cells, it is not clear what specific effect Vpr exerts on DC function and adaptive immunity. Our results based on DC-HIV infection indicate that Vpr is expressed in infected DC and that the protein specifically regulates the costimulatory and maturation markers that are normally upregulated during DC maturation without altering the other cellular proteins. Our analysis of the expression of costimulatory molecules in infected versus uninfected populations showed downregulation of these molecules in both infected and uninfected populations, though the effect was significantly greater in the infected population. This could be due to the exposure of the uninfected DC to virion-associated Vpr. There are diverse sources of Vpr available within the infected population. Upon infection of cells by HIV-1, Vpr is synthesized as a late protein, along with the structural proteins (11). Vpr is also associated with virus particles, which enables the virus particles to bring Vpr into cells upon infection. In addition to the infectious particles, there is an abundance of noninfectious particles (on the order of 1:50,000 to 1:100,000 infectious versus noninfectious), which also contain Vpr. Hence, noninfectious virions could transfer Vpr protein into cells via endocytosis, a known DC function. Additionally, the intrinsic ability of Vpr to traverse the cell membrane, as demonstrated by several groups (40, 45), provides another avenue by which Vpr may be released from the infected DC and could thereby influence the uninfected DC population. Vpr has been reported to be an immunosuppressive molecule and is known to inhibit the immune response for the codelivered antigen (4, 39). The exact mechanism of Vpr action, however, is unclear. Here, we present the first line of evidence implicating the possible mechanism(s) that could be utilized by Vpr in order to suppress the effective induction of host immune response through interfering with maturation and costimulatory molecules. We further show that the observed dysregulation of DC in the context of Vpr is not due to apoptosis. Previous reports have established proapoptotic and antiapoptotic roles of Vpr based on the source of Vpr (extracellular free or virion associated) being presented to the cells (14, 66). HIV-1 $vpr^+$ infection of DC 4 days prior to evaluation of maturation did not lead to any detectable induction of apoptosis. These findings are similar to those of Mirami et al. (38), indicating that Vpr potentiates GR-mediated immunosuppression of PBMCs independently of apoptosis.

A significant reduction of mRNA expression was observed for CD80, CD83, and CD86 in DC infected with HIV-1 $vpr^+$ compared to HIV-1 $vpr^-$ or an uninfected control. This indicates that Vpr induces downregulation of costimulatory and maturation markers through their suppression at the transcriptional level. This is different than the posttranslational degradation of CD4 and MHC class I by HIV-1 Nef (12, 13). Our report is in agreement with published results suggesting that PBMC treated with recombinant Vpr downregulated the expression of immunoregulatory genes at the mRNA level (38). At this point, it is not clear how Vpr regulates the expression of these genes. However, based on the selective inhibition of certain cellular genes, it is possible to predict that Vpr might be acting on certain transcriptional elements at the promoters of these genes, which requires further analysis. The potential candidates are the GRE, NF-κB, Ap1, and Sp1 transcription factors that are known to be regulated by HIV-1 Vpr (37, 59, 62).

In addition to the costimulatory molecules, the nature of the cytokines released during DC activation also plays a key role in determining the outcome of the T-cell response (32). The cytokine response to invading microorganisms is critical for priming the DC-mediated adaptive immune response and is subject to tight regulation, particularly in the case of the Th1-polarizing cytokine IL-12 (57, 31). During acute HIV infection, the cytokine response is disrupted, but the mechanism is poorly understood. Moreover, the deficit in adaptive immune response seen in HIV-1-infected patients is characterized by impaired cytolytic activity of $CD8^+$ T cells (63). One mechanism of this immune dysregulation is a selective inhibition of monocytes, macrophages, dendritic cells, and Th1 lymphocytes and their cytokine networks, which eventually drives viral replication. HIV infection involves an immune escape mechanism that often fails to control viral replication. Several cytokines, including IL-10, are increased during HIV replication, but IL-12 production is decreased (21, 35, 46). HIV-1-infected DC differentially regulated Th1 and Th2 cytokine production to impair host protective antiviral immunity and facilitate viral replication. Upregulation of Th2-promoting cytokine IL-10 production and suppression of Th1-promoting IL-12 have been documented in HIV-1-infected DC (21, 35). Our results show that Vpr selectively suppressed the production of cytokine IL-12 but not IL-6 upon CD40L stimulation. The deficient production of inflammatory cytokines by HIV-1 $vpr^+$-infected DC and upregulation of IL-10 found in the present

study further confirm the role of this protein in host immune dysfunction. HIV-1-infected DC are known to polarize toward immune-suppressive or tolerogenic DC (21); however, it is not clear how the surface events regulate cytokine networks in the context of HIV-1 infection of DC. Our results showed that *vpr*$^+$ virus-infected DC are deficient in Th1 cytokine production, which could be due to either defective maturation or the augmented expression of receptor molecules in association with defective signaling, like activation of ERK1/2 and inhibition of p38 MAPK, as documented in other infection systems. Consistent with this Th1-deficient phenotype, a marked reduction of TLR-4 mRNA was observed in HIV-1 *vpr*$^+$-infected DC compared to HIV-1 *vpr*$^-$-infected DC (data not shown). Similar results were observed by Mirami et al. (38) in Vpr-treated PBMC in the presence of glucocorticoid.

Mature DC are able to present processed peptides as a complex with MHC class I and costimulatory molecules on their surfaces. This event is critically required for an efficient antigen-specific T-cell response represented by a two-signal model within the immunological synapse (15). Loss of DC costimulatory molecules (CD80 and CD86) has been evident during acute HIV infection, raising the speculation that the virus disarms the host adaptive response primarily through impairing DC maturation and antigen-specific T-cell response. Both adults and children infected with HIV-1 have been known to have impaired immune responses to recall and neoantigens, and this effect has been seen as early as 3 months following seroconversion (53). Our results further confirm that the presence of Vpr could be partially responsible for this immune-suppressive effect, alone or in conjunction with other viral proteins. The impaired antigen-specific T-cell activation against recall antigen observed in this study could be due to downregulation of CD80, CD83, and CD86, together with deficient production of proinflammatory cytokines and Th1-derived IFN-γ, critically required for the activation of cellular and immune responses. Though our analysis focuses on the effect on DC, similar effects could be exerted on T cells, and this deserves further analysis.

Our findings presented here further delineate the pathways and/or immunomodulatory molecules that are disrupted by HIV-1 Vpr and that in turn might aid the virus in evasion of the host immune response. In conclusion, we have demonstrated that HIV-1 Vpr suppresses the phenotypic and functional features of DC, as evidenced by its role in upregulating the Th2 cytokine IL-10 and downregulating the Th1 cytokine IL-12, along with impaired CD8-mediated IFN-γ production, exploiting a strategy to reciprocally augment this arm of antiviral immunity. Based on the relevance of Vpr, present as both a virion-associated and cell-associated molecule in vivo, this could have effects at multiple levels. It is worth noting that other HIV-1-encoded viral proteins are also known to play important roles in immune-evasive strategies. However, it is not clear how these effects, mediated by several viral gene products, could work together in vivo. Delineating these functions and pathways and their roles in immune escape will enable further improvement in combating viral infection, including vaccine strategies against HIV-1.

## ACKNOWLEDGMENTS

HIV-1 pNL43.HSA R$^+$ E$^-$ and pNL43.HSA R$^-$ E$^-$ plasmids, as well as pVSV-G Env plasmids, were obtained from the AIDS RRRP, NIH. We thank Walter Storkus, University of Pittsburgh, and Michelle Calos, Stanford University, for gp100 T-cell clones and 293T cells, respectively. We thank John Kappes, University of Alabama, for the Vpr antibody. We thank M. Bajcz for her excellent technical assistance in generating the tyrosinase CTL clones. We thank Phalguni Gupta for his constant support and help with the cytokine array analysis and Paolo Piazza for critically reading the manuscript.

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