HIV-1 Vpr inhibits the maturation and activation of macrophages and dendritic cells in vitro

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Abstract

Human immunodeficiency virus-1 (HIV-1) Vpr encodes a 14 kDa protein that has been implicated in viral pathogenesis through in vitro modulation of several host cell functions. Vpr modulates cellular proliferation, cell differentiation, apoptosis and host cell transcription in a manner that involves the glucocorticoid pathway. To better understand the role of HIV-1 Vpr in host gene expression, ~9600 cellular RNA transcripts were assessed for their modulation in primary APC after treatment with a bioactive recombinant Vpr (rVpr) by DNA micro-array. As an extracellular delivered protein, Vpr down-modulated the expression of several immunologically important molecules including CD40, CD80, CD83 and CD86 costimulatory molecules on MDM (monocyte-derived macrophage) and MDDC (monocyte-derived dendritic cells). Maturation of dendritic cells (DC) is known to result in a decreased capacity to produce HIV due to a post-entry block of the HIV-1 replicative cycle. Based on the changes observed in the gene array, we analyzed maturation of DC generated from monocytes in tissue culture as influenced by Vpr. We observed that Vpr-treated immature MDM and MDDC were unable to acquire high levels of costimulatory molecules and failed to develop into mature DC, even in the presence of maturation signals. These studies have importance for understanding the interaction of HIV with the host immune system.

Introduction

Antigen-presenting cells [macrophages/dendritic cells (DC)], in addition to being a major target/reservoir for viral infection, play an important role in activating the immune system. DC are the critical antigen-presenting cells (APC) for the induction of primary immune responses, due to: (i) their high efficiency in capturing antigen; (ii) their ability to process antigen; (iii) their migratory capacity; and (iv) their abundant expression of molecules necessary for T cell activation and expression (1). In addition, macrophages and dendritic cells are known to express the CD4 receptor and are susceptible to infection with HIV-1. These cells also express the major HIV co-receptors CCR5 and CXCR4 and are believed to act as reservoirs for virus dissemination (2). Furthermore, APC function is critical for initiation and maintenance of the T cell response. Accordingly, understanding the effects of HIV gene products on dendritic cell biology is likely to provide important information into immune pathogenesis of HIV infection. The generation of antigen-specific cytotoxic T lymphocytes is thought to be essential for controlling HIV infection (3,4). In fact, there is an inverse correlation between viral load and CTL potency, suggesting that T cell activation is crucial for limiting viral propagation (5). Therefore, understanding how antigen presentation is modulated by the genes of HIV-1 is important for understanding viral pathogenesis.

In this study, we have focused on the modulation of APC function by the HIV-1 accessory protein Vpr. This HIV viral antigen modulates numerous cellular events, including cell cycle arrest, differentiation, apoptosis, NF-κB suppression, nuclear herniation and nuclear migration (6–15). Previously, we and others have reported that Vpr suppresses the NF-κB pathway by binding to and signaling through the glucocorticoid receptor complex (13,16). This pathway is thought to attenuate innate immune recognition by monocytes and suppress β-chemokine production by macrophages (17,18). However, the direct effect of Vpr on macrophages/DC to regulate T cell activation has not been investigated. This is of
HIV-1 Vpr modulation in antigen-presenting cells

importance, as HIV targets APC for dissemination into the lymph nodes for T cell infection and because of Vpr’s transducing capability of cells (19,20). Therefore, we implemented a comprehensive micro-array analysis of recombinant Vpr (rVpr)-treated PBMC (peripheral blood mononuclear cells) to observe Vpr’s effect on transcriptional profiles. Interestingly, we ascertained that essential costimulatory molecules including CD40, CD80 and CD86 are severely diminished in cells treated with rVpr. Also, administration of rVpr at picogram levels was sufficient to impede the maturation of DC and down-regulate the surface expression of MHC Class II molecules. Our data suggest that this inhibition of maturation of APC inhibition directly hinders presentation and proliferation of T cells. Furthermore, Vpr treatment of Vpr-treated APC was sufficient to provoke a poor response phenotype, likely due to poor antigen presentation without costimulation.

Methods

Reagents and antibodies

Recombinant human (rh)IL-4, rhTNF-α, rhGM-CSF and anti-CD3 mAbs, and conjugated human mAbs to CD1a, CD3, CD4, CD8, CD14, CD33, CD80, CD83, CD86, HLA class I, DC-sign and MHC-II DR and their matched isotypes were purchased from BD Biosciences Pharmingen (San Diego, CA). PHA was purchased from Sigma Chemical Co. (St Louis, MO).

Preparation of T cells from peripheral blood mononuclear cells

PBMC were obtained using Ficoll-Hypaque™ Plus density gradient centrifugation (Amersham Pharmacia LKB, Piscataway, NJ) purchased from the Human Immunology Core/CFAR Immunology Core, University of Pennsylvania School of Medicine. Cells were subsequently grown in RPMI with 10% FBS supplemented with 10% FCS in aliquots at 80 g of pEnv (VSV-G envelope: vesicular stomatitis virus glycoprotein envelope), or 10 µg of pNL4-3.HSA.R+ with 5 µg of pEnv (VSV-G envelope) and 5 µg of pVpr constructs in 293T cells (American Type Culture Collection, Rockville, MD). For preparation of infectious viral stocks, 293T cells were plated on 100 mm tissue culture plates and transfection was done using DOTAP (Roche Applied Science, Indianapolis, IN). At 6 h post-transfection, the medium was replaced with fresh DMEM supplemented with 10% FBS. At 72 h after transfection, the virus containing supernatants were collected and then centrifuged for 10 min at 1500g to remove cells, passed through 0.4 µm-pore-size filters and ultracentrifuged at 69,000g for 90 min. Supernatants were collected and resuspended in 0.1× Hanks balanced salt solution (HBSS) and the viral titers were determined by p24 ELISA assay kit (Beckman Coulter, Miami, FL) as well as p24imm staining by FACS analysis. The viral stocks were stored in the presence of 10% FCS in aliquots at −80°C. Infection was performed by incubating target cells with appropriate virus at a concentration of 100 TCID50/10⁶ cells/ml (12).

Recombinant Vpr protein purification

HIV-1 vpr gene was PCR amplified using the following Vpr primers: Vpr (+) 5’-ACGGATCCATGGAAACGCCCAGA-3’; Vpr(−) 5’-TGGATCTACTGGCTCCATT-3’; and cloned into pTYB4 vector (New England Biolabs, Beverly, MA). Highly purified (>95%) recombinant Vpr protein (pTYB4-Vpr) was prepared by using the IMPACT-CN system (New England Biolabs, Beverly, MA) for treatment of the cells. Eluted protein was analyzed by SDS-PAGE gel to assess its purity. The Vpr was found to be 99% pure. HIV-1 Gag (p55) protein obtained through NIH AIDS Research and Reference Reagent Program (NIH-AIDS-RRRP) was used as a control in our assay. This method of purification has been previously described by several groups as reproducing Vpr's native activity (22). To establish dose profiles, Vpr protein was used at 0, 10, 100 and 1000 pg/ml concentrations. Normal human PBMC from healthy human donors were purified by Ficoll-Paque™ density 1000 U/ml rhGM-CSF and 500 U/ml rhIL-4. At day 5, non-adherent cells were rinsed off, washed once in PBS, and transferred to 6-well plates at 7 × 10⁵ cells in 3 ml/well. The resulting cell preparation contained >90% DC as assessed by morphology and FACS analysis.

For differentiation into mature DC (mDC), immature DC (iDC) were additionally stimulated on day 6 with 10 ng/ml rTNF-α. Immature DC typically were HLA-DR++, CD86++, CD83-weak and CD14weak, whereas mature DC were HLA-DR++, CD86++++, CD83+++ and CD14+. All cytokines used in this study were recombinant human proteins and were used at plateau concentration to induce the optimal generation of DC.

Construction and generation of HIV-1 Vpr molecules

The HIV-1 clone pNL4-3 HSA.R+ E− or pNL4-3 HSA.R+E− were obtained from the NIH AIDS Research and Reference Reagent Program (NIH-AIDS-RRRP). These viruses have been rendered env and vpr deficient by introduction of frame shifts and therefore could only generate replication-incompetent virions (7). HIV-1 virions containing Vpr are established by co-transfecting 10 µg of pNL4-3.HSA.R+ with 5 µg of pEnv (VSV-G envelope: vesicular stomatitis virus glycoprotein envelope), or 10 µg of pNL4-3.HSA.R− with 5 µg of pEnv (VSV-G envelope) and 5 µg of pVpr constructs in 293T cells (American Type Culture Collection, Rockville, MD). For preparation of infectious viral stocks, 293T cells were plated on 100 mm tissue culture plates and transfection was done using DOTAP (Roche Applied Science, Indianapolis, IN). At 6 h post-transfection, the medium was replaced with fresh DMEM supplemented with 10% FBS. At 72 h after transfection, the virus containing supernatants were collected and then centrifuged for 10 min at 1500g to remove cells, passed through 0.4 µm-pore-size filters and ultracentrifuged at 69,000g for 90 min. Supernatants were collected and resuspended in 0.1× Hanks balanced salt solution (HBSS) and the viral titers were determined by p24 ELISA assay kit (Beckman Coulter, Miami, FL) as well as p24imm staining by FACS analysis. The viral stocks were stored in the presence of 10% FCS in aliquots at −80°C. Infection was performed by incubating target cells with appropriate virus at a concentration of 100 TCID50/10⁶ cells/ml (12).

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gradient centrifugation as previously described. Cells (1 × 10⁶) were treated with different concentrations of rVpr protein or control protein (rGag) for 6 h, washed with PBS and resuspended in RPMI medium with 10% FBS. To confirm the specificity of our antibody, Vpr antisera was added to the cells at various dilutions (1:10 to 1:10 000) and then assayed. Negative controls were mock (PBS)-treated.

cDNA micro-array analysis and data analysis

Vpr-induced differential expression of host cellular genes was performed by cDNA micro-array as described (23). Total cellular RNA was extracted using the RNAagents RNA isolation kit (Promega, WI). After precipitation with isopropanol, the RNA was rinsed with 70% ethanol; air dried and resuspended in diethyl pyrocarbonate (DEPC)-treated water and stored at −20°C. Total RNA concentration was measured by absorption spectrophotometry at 260 nm and the A₂₆₀/A₂₈₀ ratio averaged 1.7–1.8. Human GDA (1.3) cDNA micro-array membranes were purchased from Genome systems (Incyte Genomics Inc). This array consisted of 9600 individual spots, each representing a known human gene. Poly (A)+ RNA was purified from various experimental cells and 1 µg of mRNA was used as a template for the preparation of cDNA and amplified mRNA (aRNA) from the rVpr-treated and mock cells were prepared according to the method described by Kacharmina et al. (23). To generate gene expression profiles of mRNA populations, radiolabeled aRNA was used to probe for reverse northern blots containing several thousand candidate cDNAs. The radiolabeled blots were then dried and placed in a PhosphorImager (Molecular Dynamics) cassette for 24 h. Quantitation of hybridization intensity was performed by scanning densitometry. Ratios of mock- to rVpr-treated (or vice versa) intensities were calculated to compare the relative expression levels of genes between the experimental and reference samples. These results were normalized using a global median gene expression value computed for each array. To improve the reliability of the data, weak signal spots were removed according to the following criteria. The ratios for potential differentially regulated genes were calculated after correction for probe variation using constitutively expressed genes on equivalent arrays (24). Any genes calculated to have a differential expression of less than 2-fold were disregarded. This very stringent cutoff value was set to eliminate false positives, but it should be noted that doing so may have excluded some genes that were differentially expressed by low, but biologically significant amounts.

Northern blotting analysis

For the northern blotting analysis, 10 µg of total RNA underwent electrophoresis in 1.2% agarose formaldehyde gels, which were blotted onto a nylon membrane. The filters were hybridized using [α-³²P]dCTP-labeled CD33 cDNA as a probe by the random primed labeling DNA kit (Promega, Madison, WI). In brief, the filters were hybridized with labeled probes (1 × 10⁶ c.p.m./ml) in buffer (40% formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt’s solution, 40 µg/ml sonicated and denatured salmon sperm DNA, 0.1% SDS, 20 mM Tris pH 7.5) at 42°C for 16 h. The filters were washed twice for 15 min at room temperature and once for 30 min at 56°C with 2× SSC containing 0.1% SDS, and then exposed using a Kodak Imaging plate (25).

Flow cytometric analysis

For FACS staining, single cell suspensions were washed in PBS (pH 7.2) containing 0.2% bovine serum albumin and 0.1% Na₂SO₃. Cells were incubated with goat IgG to block binding of Ig to FcβR and stained with FITC or PE-labeled specific mAb diluted to the optimal concentration for immunostaining for 30 min at 4°C, washed three times and analyzed by flow cytometry. Dead cells were excluded from the analysis by propidium iodide staining and live cells were gated on the basis of their forward scatter and side scatter characteristics and analyzed directly on a Coulter EPICS Flow Cytometer (Coulter, Hialeah, FL) using FlowJo software (TreeStar, San Carlos, CA). All samples were compared to their isotype-matched controls. In the case of dual flow cytometry individual samples treated with each isotype alone were used to determine the background levels of auto fluorescence.

Phagocytosis assay

T cells and MDM were isolated from PBMC of healthy donor as described above. We analyzed the in vitro flow cytometric technique for the quantification of phagocytosis ability by macrophages as described before, but with modifications (26). Before the phagocytosis assay, purified T cells (cell count adjusted to 2 × 10⁶ cells/ml) were washed with RPMI-1640 three times and apoptosis was induced by adding 500 nM dexamethasone (Dex) for 6 h incubation at 37°C. Cells were centrifuged at 500 g for 5 min and resuspended in 10 ml culture medium. The apoptotic T cells were stained with 50 µl MTG (25 µg/ml) (Molecular Probes, OR) for 15 min and then washed twice in Hanks buffer. Positively stained cells were resuspended in culture medium and added to the purified MDM, which were cultured in 6-well plates in RPMI-1640 medium treated with PBS (mock), rGag (10 µg/ml) or rVpr (10 µg/ml) for 2 h. The labeled T cells were resuspended in RPMI-1640 and added to the plates with MDM at a ratio of 10:1 T cells to MDM, and incubated for 12 h. The plates were washed with RPMI-1640 to remove bound T cells. The MDM in the plates were washed with PBS, resuspended in 200 µl PBS and analyzed for Mitotracker–FITC expression directly on a Coulter EPICS Flow Cytometer (Coulter, Hialeah, FL) using FlowJo software (TreeStar).

Immunostimulatory capacity of DC

Monocytes were isolated from the PBMC, and subsequently, these monocytes were used to generate DC. Mature DC were pretreated with rGag or rVpr (10 µg/ml) for 4 h. Mixtures of rVpr- and rGag-treated cells were as follows: rGag-treated DC only; 7/8 rGag-treated DC and 1/8 rVpr-treated DC; 3/4 rGag-treated DC and 1/4 rVpr-treated DC; or rVpr-treated DC only. Graded numbers of each mixture and 10⁵ purified, allogeneic T cells (as designated) were incubated in 96-well flat-bottom plates. To test the immuno-competency of T cells, two batches of 10⁵ mature DC were treated with either rGag or rVpr (10µg) for 4 h, and then each incubated with 10⁷ allo-lymphocytes for 2 days. The DC were then removed using plates coated with antibodies to CD83 and CD86. Remaining lymphocytes were
then confirmed for the lymphocytes population by FACS analysis and split into four batches. Two of these batches were pre-incubated with rVpr-treated DC and the other two were pre-incubated with rGag-treated DC. These four batches of lymphocytes were then again challenged with graded doses of DC from two other batches of 10⁶ mDC from the same donor, which had been pretreated with either rGag or rVpr for 4 h. After 6 days, the cells were pulsed with 1 μCi of [³H]thymidine (NEN Life Science, Boston, MA). The cells were harvested onto filters and radioactivity was measured in a scintillation counter 24 h later. Each culture condition was tested in triplicate and the mean counts per minute (c.p.m.) were calculated. For background control, [³H]thymidine incorporation of T cell or DC with medium alone was measured in each experiment (27). All the experiments were performed at least three times, and the data from one representative experiment are shown.

Quantitation of secreted cytokines by ELISA
We measured the level of cytokines present in the supernatant using capture ELISA. We measured IL-12 and TNF-α cytokines from the culture supernatant. Recombinant proteins were used as a standard, and capture and detection antibodies were purchased from R&D Systems (Minneapolis, MN). The assay was performed according to the manufacturer’s protocol. Briefly, 96-well ELISA plates were coated with capture antibodies (10 μg/ml) overnight at 4°C. Plates were washed 1× with PBS and blocked with 2% BSA for 1 h at 37°C to reduce non-specific binding. Supernatants from the infected cell cultures were added to the wells in triplicate and the mean counts per minute (c.p.m.) were calculated. IL-12 and TNF-α cytokines were detected at 450 nm in an ELISA plate reader.

Results

Vpr purification and functional assay
HIV-1 Vpr (89.6) was cloned into the pTYB4 expression vector using DNA fragments amplified by PCR from a proviral template. The purified fragment was inserted between the NcoI and SmaI sites in the pTYB4 vector. Final constructs were sequenced and confirmed to be authentic Vpr. Vpr was expressed by using bacterial fusion expression system. The cleavage product was further purified by metal affinity column purification, followed by dialysis at 4°C to ensure complete removal of any salts and contaminations. Purified bacterial cell-derived Vpr protein showed a single band with a molecular weight of 14 kDa detected by SDS-PAGE gel following western blotting with anti-Vpr antibody (Fig. 1A). Since we and others had reported that Vpr induces cell cycle arrest and apoptosis, we confirmed the functional effects of this recombinant Vpr protein on the induction of cell cycle arrest and apoptosis. In vitro extracellular rVpr or rGag (control protein) were used to examine the Vpr activity on Jurkat T cells. These experiments demonstrate that addition of this extracellular rVpr protein strongly induced cell cycle arrest at G2/M phase and apoptosis at a concentration of only 10 pg/ml (Fig. 1B). In contrast, rGag had little effect on cell cycle arrest and produced only background levels of apoptosis compared to rVpr. This activity is consistent with prior reports of Vpr activity (22,28,29).

Differential expression of host cell genes by cDNA micro-array
The expression of host cell genes in rVpr protein (10 pg)-treated human PBMC was investigated by cDNA micro-array analysis. Two days post-treatment, total RNA was isolated from the rVpr-treated, control protein-treated or mock-treated groups and mRNA was obtained from each group. Pair-wise hybridization was performed in which the expression pattern obtained with each group was compared to each mock-treated group. The 2 day time point was chosen for analysis, as this is a period post-treatment that appears to represent the height of Vpr’s effects (19,22,28–30). Each filter contains 9600 cDNA fragments representing previously characterized human genes divided into six quadrants representing different categories of genes, including the following: (i) oncogenes, and tumor suppressor gene and cell cycle regulators; (ii) stress response genes, ion channels and transport genes; (iii) apoptosis-related genes and genes involved in DNA synthesis, repair and recombination; (iv) transcriptional factors and general DNA-binding proteins; (v) cell receptors, cell surface antigen and cell adhesion molecules; and (vi) cell–cell communication factors. The raw data were collected, normalized and statistically analyzed using custom software as indicated in the Methods. We defined the threshold value for genes regulated by rVpr as being significantly induced/down-regulated when expression is at least 2-fold higher/lower than expression in the control experimental samples (Fig. 2). This analysis serves to emphasize particular major changes in gene expression and facilitates the analysis of the large quantity of data generated in this type of study.

Vpr effects on CD33 expression and phagocytosis
Among the messages that were down-regulated by HIV-1 Vpr, we observed that CD33 was strongly down-modulated at the RNA level. Antigen uptake is through an ordered process of endocytosis/phagocytosis, which starts with the docking of proteins or cells on the surface of the phagocytic cells through specific receptors, including CD33 and the macrophage scavenger receptor (31). We first sought to confirm Vpr’s effect on these important markers. CD33, as well as class-I and class-II, expression were analyzed in rVpr-treated cells by flow cytometry (Fig. 3A). There was an effect on HLA class-I and MHC-class-II expression, which can be a marker of immune activation. Similarly, CD33 expression on a large subset of rVpr-treated cells was also decreased. We hypothesized that Vpr effects on MHC class II and CD33 could impact antigen presentation. As CD33’s expression is predominantly on APC, we next examined Vpr’s modulation of CD33 on macrophages/monocytes using CD14 as a specific subset marker. In this analysis, rVpr suppressed >80% of CD33 expression on CD14 positive cells, illustrating a dominant effect of Vpr on this important marker (Fig. 3B). We confirmed Vpr’s effects on CD33 by both northern and
western blotting analysis (Fig. 3C). Overall, rVpr strongly diminished CD33 expression in PBMC, suggesting that some cells may be particularly sensitive to down-modulation by Vpr.

T cell activation requires antigen processing and presentation in the context of MHC molecules by professional APC. Since CD33 binds to many proteins and cells, and it is expressed on phagocytic cells, we wanted to test the possibility that CD33 mediates the interaction of phagocytic cells and whether these target cells or proteins are influenced by Vpr. It has been reported that apoptotic bodies are engulfed by professional APC (31). We used an in vitro flow cytometric technique to quantify phagocytic ability of macrophages, as described in the Methods section. In this assay we purified T cells and macrophages separately, and induced apoptosis in the cultured T cells that had been stained with Mitotracker Green (MTG) (Fig. 4A). The apoptotic T cells were then exposed to purified autologous macrophages that had been pre-treated with rVpr protein or rGag protein and then washed and re-cultured in tissue culture. Adherent macrophages without apoptotic T cells are negative for MTG staining while macrophages treated with apoptotic T cells bodies show positive staining for MTG (21.6%). This positive result was also observed with rGag-treated control (20.9%) macrophages. In contrast, rVpr-treated macrophages are deficient (2.51%) in their ability to phagocytose when compared to either of these controls. There was no significant difference in the amount of phagocytosis in normal macrophages (Fig. 4B). This study illustrates that Vpr can target suppression of the in vitro phagocytosis, and consequently could influence antigen presentation.

**Fig. 1.** Purification and bioactivity of rVpr. Vpr was produced as a fusion protein in bacteria using the Intein system as described in the Methods. (A) Western blotting analysis of purified rVpr protein. The fusion protein and induced Vpr cleavage product was identified by western blotting analysis using polyclonal anti-Vpr antibodies raised in rabbits. Lane 1 is mock; lane 2 is fusion protein without cleavage; and lane 3 is the cleaved purified rVpr protein. The Intein–Vpr fusion is 55 kDa, and the free Vpr is a 14 kDa protein. Standards are indicated on the left. (B) Effect of rVpr on cell cycle arrest and apoptosis. Jurkat cells (1 × 10⁶) were treated with the following: mock, rGag (10 pg/ml) or rVpr (1 pg or 10 pg/ml). 24 h post-treatment, cells were pelleted, stained with propidium iodide for cell cycle arrest and analyzed for apoptosis by annexin V staining as described in the Methods. DNA content was analyzed based on untreated controls. Relative cell numbers are indicated on the y-axis, and DNA content is indicated on the x-axis. Similarly, apoptosis was detected by flow cytometry, and the numbers shown in the histogram represent the percentage of cells with exposed phosphatidylserine, which is indicative of positive apoptosis. These results are representative of three independent experiments.
Effect of Vpr on expression of costimulatory molecules in macrophage and dendritic cells

DCs are recognized to be highly effective cells for the presentation of MHC displayed antigen to naïve T cells for induction of the primary immune response. The importance of this antigen presentation in the generation of the immune response has been extensively studied (1,2). Based on the effects of Vpr on CD33, we next sought to study Vpr’s effects on co-stimulatory molecules. For the study, MDM and MDDC were generated from PBMC as described before (21,32). The generation of the antigen-specific T cell immune response is a complex process that requires the engagement of T cells with professional APC. APC (including DC, B cells and macrophages) drive antigen-specific immune responses through up-regulation of the CD40, CD80 and CD86 co-stimulatory molecules as they interact with T cells during initial T cell activation events (33–38). We examined Vpr’s effects on co-stimulatory markers of MDM and MDDC cells treated with 10 pg/ml of rVpr. Treatment with rVpr specifically lowered expression of all three important co-stimulatory molecules on MDM, as opposed to treatment with a control rGag protein (Fig. 5A). However, there was no significant change in the level of expression of CD14+, suggesting that Vpr interferes specifically with these co-stimulatory molecules, a conclusion which is also supported by the gene chip analysis. We next examined Vpr’s effects on co-stimulatory molecule expression on the MDDC cell population (Fig. 5B). CD80 and CD86, as well as the maturation and lineage-specific marker CD83, were all down-modulated in the MDDC cell population. Some effects of Vpr on host cell biology are blocked by anti-GR compounds including RU486 (13,18). We therefore tested whether the effects of rVpr on modulation of DC could be reversed by RU486, a GR...
antagonist. In this study, we treated DC with rVpr in the presence or absence of RU486 (1 μM). In this assay, RU486 completely inhibited the ability of Vpr to down-modulate DC, suggesting that the RU486 reverts the observed Vpr phenotypes. These data suggest that in macrophage and DC, Vpr can dramatically affect molecules important in the induction of an immune response at least in part through GR modulation.

Effect of Vpr on differentiation of monocytes into DC
To determine whether Vpr would inhibit maturational changes in DC in the absence of T cells, we next stimulated immature DC (Fig. 6A) with TNF-α in the presence or absence of rVpr. Cytokines (GM-CSF, IL-4 and TNF-α), which trigger DC maturation (1,21,33), induced strong up-regulation of the costimulatory molecules CD40, CD80 and CD86, the major histocompatibility complex (MHC) class II molecule and the DC maturation marker CD83 (Fig. 6B). In the presence of rVpr at 10 pg/ml/1 x 10^6 cells, these cytokine-induced phenotypic changes were dramatically impaired (Fig. 6C). The up-regulation of CD40, CD80, CD86 and of the MHC class I and II molecules was largely inhibited and CD83 was not expressed at all. Importantly, rVpr-treated DC did not revert to the monocyte/macrophage stage as shown by the lack of expression of CD14 (data not shown). These experiments

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**Fig. 3.** (A) Modulation of CD33, HLA-class I and MHC-IIDR by rVpr in PBMC. Total PBMC were treated with rGag or rVpr protein as indicated. 24 h post-treatment, the cell surface phenotype of treated cells was examined by FACS analysis with the indicated specific antibodies. Histograms show the indicated surface markers, and filled histograms represent the isotype-matched control antibody staining. Similar results were obtained in three independent experiments. (B) Flow cytometry analysis of CD33 expression in mock-, rGag- or rVpr-treated monocytes. Purified monocyte cells were harvested 24 h post-treatment and stained with CD14 and specific anti-CD33 antibody. Histograms show the CD33 expression on CD14-positive monocytes population and filled histograms represent the isotype-matched control antibody staining. These experiments were repeated three times, and similar observations of CD33 suppression were observed. (C) Expression of CD33 in rVpr-treated PBMC. (i) Northern blotting analysis of CD33 mRNA shows down-regulation by rVpr treatment. Total RNA from the mock-, rGag- or rVpr-protein-treated cells was extracted 24 h after treatment and 10 μg of total RNA of each sample was subjected to electrophoresis in 1.2% agarose formaldehyde gels. The RNA gel was then blotted and transferred onto a nylon membrane and hybridized with a CD33 cDNA probe as indicated in the Methods. Afterwards, the membrane was reprobed with GAPDH for comparison with a housekeeping mRNA. (ii) Western blotting analysis of CD33 expression in rVpr-treated PBMC. Samples were treated as described above and cells were lysed as indicated in the Methods, then the proteins were separated by 12% SDS–PAGE and blotted. The resulting blots were analyzed using anti-CD33 mAb as a specific probe or for actin expression using an anti-actin antibody as a control. The arrow indicates the 67 kDa protein of CD33 expression.
confirm that HIV-1 Vpr prevents phenotypic changes induced by cytokine signals important in the maturation of immature DC.

**Inhibition of antigen-specific CD4 T cell responses by Vpr**

*In vivo* Vpr can interfere with the *de novo* immune response generation both in mice (30) and in non-human primates (39). The mechanism(s) by which Vpr suppresses these CD4+ T cell responses are still unknown. It is clear from the results above that rVpr-treated APC are defective for expression of important costimulatory molecules necessary in antigen presentation. In fact, presentation of antigen in the absence of co-stimulation could result in depletion of reactive T cells or poor responsiveness (34). To investigate whether rVpr-treated APC could generate an inhibitory (immunomodulatory) effect, we cultured T cells with a mixture of rVpr-pretreated or control protein (rGag)-pretreated DC. We observed that the presence of as little as 12.5% (1/8) or 25% (1/4) rVpr-pretreated DC resulted in a 5- to 10-fold reduction in the resulting immune response, as judged by the slope of the proliferative response curve at day 6 (Fig. 7A). This result led us to investigate whether this was in fact a form of T cell anergy. Accordingly, we attempted to restimulate T cells which had been exposed to rVpr-treated DC or rGag-pretreated DC from the same donor by removing rVpr-treated DC or rGag-pretreated DC through panning of the T cell/DC culture with anti-CD83 and anti-CD86 (hence removing DC). The T cells were then washed and confirmed to be positive for the lymphocyte population markers and depleted of DC by FACS analysis using specific markers. These T cells were re-incubated with DC pulsed with antigen to ascertain the tolerance or the lack of immunogenicity induced. The results show that T cells exposed for 2 days to rVpr-treated DC could not proliferate upon challenge with rGag protein (Fig. 7B).

The pattern of cytokine expression influences the nature and persistence of the inflammatory response central for the induction of cellular immunity. Supernatants from the above experiment were collected and assayed by ELISA for TNF-α and IL-12 production. Addition of rVpr significantly inhibited >3-fold production of inflammatory cytokines, as measured in the supernatants of these cultures (Fig. 7C). These results suggest that Vpr’s effects on APC can have a profound effect on the activation and generation of antigen-specific T cells. Taken together, these data suggest that Vpr blocks the processing, and/or presentation signals of APC that are likely to be critical for induction of the adaptive arm of the T cell response.

**Effect of Vpr as virion-associated molecule on expression of costimulatory molecules**

We next sought to confirm if Vpr as a viral borne molecule could similarly influence immune response induction as was observed with the recombinant Vpr protein. We evaluated the
effect of a virion-associated Vpr molecule on expression of co-stimulatory molecules on APC by infecting macrophages using VSV-complemented pseudotype HIV-1 Vpr+/Vpr− viruses. VSV-complemented HIV-1 pseudovirus allows analysis of single round replication, facilitating analysis of the effect of viral proteins without subsequent viral spread. Vpr-positive virus infection of macrophages down-modulated expression of CD40, CD80, CD83 and CD86, compared to uninfected or HIV-1vpr− virus-infected cells (Fig. 8). We show that both viral Vpr and recombinant Vpr protein are capable of inhibiting the maturation of APC. Viral borne Vpr-induced down-regulation of these important molecules supports that Vpr in the context of infection may interfere with the early interactions between infected APC and T cells necessary for an effective immune response to HIV-1.

Discussion
Cellular immunity, specifically MHC-restricted CTL responses, are thought to play an intrinsic role in protection and clearance of many viral infections. Though HIV-1 infection is controlled by the immune response initially, the immune system fails to clear
Fig. 6. Effect of rVpr on DC maturation. Immature DC were generated from PBMC as described in the Methods. (A) Fresh immature DC were resuspended in culture medium supplemented with GM-CSF (1000 U/ml), IL-4 (500 U/ml) and 10 ng/ml of TNF-α, and they were allowed to adhere to 6-well plates in the absence (B) or presence (C) of rVpr (10 pg/ml) for 7 days to develop into mature DC for 7 days. On day 0 and 7, the cells were harvested, washed and analyzed for surface expression using the indicated markers by flow cytometry. Histograms show the staining of specific surface markers, and filled histograms represent the isotype-matched control antibody staining. The results are representative of three independent experiments.
the virus and ultimately loses control of viremia through unclear mechanisms (3,4,40–45). Furthermore, there is evidence that the host immune response is compromised early in HIV infection. A possible important player in the compromised immune control of HIV could be the HIV accessory antigen Vpr. HIV-1 Vpr exerts significant effects on cellular proliferation, differentiation, regulation of apoptosis, modulation of cytokine production and suppression of host cell-mediated NF-κB transcription (6–14) in vitro. Many of these Vpr-mediated cellular events have been observed in a wide variety of cell lineages, suggesting that Vpr targets basic eukaryotic cellular pathways. Previously, we and others have shown that Vpr also exerts robust anti-inflammatory effects (13,17,18,46,47). Additionally, co-immunization of pVpr with other HIV antigens diminishes their immune potency and limits vaccine control of viral load (39). Therefore, it is likely that Vpr could inhibit the cellular immune response within an HIV infection setting. However, the potential effects of Vpr on macrophage/APC remain elusive. For instance, it remains unclear whether decreased antigen-specific T cell proliferation is a result of aberrant antigen presentation or if it is a direct effect on the T cell. This is especially important, as macrophages and dendritic cells are thought to be responsible for transporting the virus to the T cells.

In this study, we investigated the role of Vpr, as a recombinant protein on immune cell transcriptional activation. We first examined the expression of genes regulated by Vpr through treating human PBMC with purified rVpr protein and analyzing transcription using a standard micro-array analysis. The micro-array analysis suggested that rVpr treatment effects molecules important in co-stimulation and immunological cell activation markers. These findings suggest that Vpr could interfere with DC functions or maturation, or both. Similarly, we observed that Vpr affects the expression of the CD33 antigen. CD33 is a 67 kDa transmembrane surface protein and its expression is specific for myeloid lineage cells. CD33 expression is higher in
CD33 belongs to the family of immunoglobulin-like lectins. Its extracellular domain has two immunoglobulin-like domains, and it binds to sialic acid (N-acetylneuraminic acid), which is present in many secreted or membrane proteins. Its cytoplasmic domain has two tyrosines whose phosphorylation results in the recruitment of molecules, which suppress cell activation. Because of the existence of sialic acid in many secreted or membrane proteins, and because these membrane proteins exist on virtually all types of cells, potentially, CD33 is able to interact with many secreted proteins and many different cells (48,49). This activity makes CD33 a likely important player in antigen presentation. The gene chip analysis results were supported by the effects of Vpr on these important antigens as determined by flow analysis. The results suggest that Vpr modulates CD33 expression differentially in PBMC versus monocytes/macrophage populations. Accordingly, further study of the effects of Vpr on APC will be important.

Co-culturing of monocytes and dendritic cells with rVpr in conjunction with TNF-α abolished APC maturation. This effect was entirely due to rVpr, as addition of anti-Vpr antibody abrogated the observed Vpr effects. The potential consequences of failed maturation of APC during HIV infection could be significant. For instance, CD40 functions by interacting with CD40 ligand (CD40L) expressed on activated CD4+ or CD8+ T cells. The engagement of CD40L on APC with or without T helper cells, have been shown to ‘condition’ the APC for antigen-specific CTL activation, and facilitates induction of a memory T cell response (35–38,50,51). Therefore, the engagement of CD40 with its ligand becomes advantageous for activation of CTLs during an immune response. Vpr-mediated CD40 repression could contribute to the inability of the host immune system to continue to mount an effective response against HIV.

Among the different costimulatory molecules, the B7 molecules (CD80 and CD86) have been observed to provide potent immune signals for T cell activation. They bind to their receptors (CD28/CTLA-4) present on T cells, first activating and then turning off the immune response (33). The CD80 and CD86 molecules are surface glycoproteins and members of the immunoglobulin superfamily, which are expressed only on professional APC (33). Blocking of these costimulatory signals leads to T cell non-response (31,51). Therefore, in addition to CD40, the overall reduction of B7 expression could contribute to attenuation of APC function in HIV infection. It is interesting to note that similar findings of lower levels of costimulatory molecules have also been observed in vivo within the lymphoid tissues of HIV-infected patients. During an acute phase infection, an increase in DC with reduced CD80/86 was
reported (37,38). Therefore, despite efficient migration of APC into regional lymph nodes, Vpr's effects may influence both presentation and activation of T cells, thus influencing viral clearance. This unique property of Vpr could ensure infection in a T cell rich environment without immune clearance and favor viral propagation.

In order to ascertain the effects on T cell activation by Vpr driving APC dysfunction, we pulsed rVpr-treated DC with rGag protein and measured T cell proliferation and immune modulatory cytokine production after removal of Vpr through in vitro co-culture experiments. Our results indicate that T cell proliferation, and more importantly cytokines, were severely diminished as a result of rVpr treatment of DC. This indicates that Vpr could impact the development of anti-HIV-specific CTLs through indirect suppression of APC function. Previous evidence also suggests that T cells from asymptomatic HIV-1 positive individuals seem non-responsive and have an attenuated capability to produce IL-2. In fact, it has been observed that defective T cell presentation may be the responsible factor (51). Furthermore, Dybul et al. have previously described roles for CD40 signaling as the mechanism for stimulating hyporesponsive p24-specific T cells, suggesting that aberrant CD40 signaling is likely a contributor to HIV-induced anergy (52).

In conclusion, our results suggest that Vpr can affect dendritic cell/macrophage maturation and activation in vitro. This effect could in vivo lead to diminished T cell activation and consequently T cell non-responses, providing an additional mechanism for HIV's evasion of the host's immune system. Further study of this issue is likely warranted.

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Abbreviations

APC: antigen-presenting cells
DC: dendritic cells
HIV: human immunodeficiency virus
iDC: immature DC
mDC: mature DC
MDM: monocyte-derived macrophages
MDDC: monocyte-derived dendritic cells
MTG: mitotracker green
PBMC: peripheral blood mononuclear cells
rVpr: recombinant viral protein R
Vpr: viral protein R
VSV-G: vesicular stomatitis virus glycoprotein

References


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