# TRANSCRIPTIONAL REGULATION OF TUMOR NECROSIS FACTOR-*alpha* BY HUMAN IMMUNODEFICIENCY VIRUS-1 VPR

by

**Shaylee M. O'Leary**

BS in Biology, The Pennsylvania State University, 2006

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This thesis was presented

by

**Shaylee M. O'Leary**

It was defended on

June 2, 2008

and approved by

YuanPu Di, PhD, Assistant Professor, Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Jeremy Martinson, PhD, Assistant Professor, Infectious Diseases and Microbiology Graduate School of Public Health, University of Pittsburgh

**Thesis Director**: Velpandi Ayyavoo, PhD, Assistant Professor, Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

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Shaylee M O'Leary, M.S.

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HIV-1 Vpr is known to regulate both viral and host cellular promoters resulting in transcriptional regulation of various cellular factors in host immune cells, such as T cells, macrophages and dendritic cells. It has been shown that Vpr has a role in the upregulation of proinflammatory cytokine TNF-α, which affects immune regulation during infection. However, the mechanisms by which TNF-α is regulated by HIV-1 Vpr are not well understood. A goal of this project is to determine the effects of Vpr in its biologically relevant forms and identify the domains of Vpr involved in TNF-α production. Additionally, we also sought to determine whether TNF-α is up-regulated in infected/exposed cells and/or bystander cells. From our experiments, we conclude that HIV-1 Vpr increases TNF-α production in the context of infection as well as exposure in the absence of other viral proteins. Furthermore, HIV-1 Vpr has multiple domains capable of inducing TNF-α production. However, the increase in TNF-α production in DC is dependent on LPS stimulation. We were unable to conclusively determine the cell type that is responsible for this observed phenotype however the results from our studies indicate that infected/exposed cells could be the dominant producers.

Due to the association of Vpr with transcriptional regulation of various cellular factors, we investigated the domains of the TNF-α promoter involved in Vpr-mediated TNF-α regulation. Using the HeLa T4 cell line, TNF-α promoter mediated transactivation was increased by two

fold when exposed to HIV-1 Vpr(+) as opposed to HIV-1 Vpr(-) as detected by luciferase reporter assay. A six fold increase was observed in the transactivation of full length and mutant TNF-α promoter in macrophage-derived microglia cell line in the presence of Vpr expression. Results from mapping studies indicate that HIV-1 Vpr can regulate TNF-α production via multiple domains of the TNF-α promoter, however for maximum transactivation, the full-length promoter is required.

Statement of Public Health Significance: By determining the details of HIV-1 Vpr and TNF-α interaction and the mechanisms for which they interact could reveal novel targets for the development of HIV-1 therapeutics in the fight against this epidemic.

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# 1.0 CHAPTER ONE: INTRODUCTION

## 1.1 THE AIDS PANDEMIC

HIV/AIDS (Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome) is a disease that is now pandemic throughout the world. As of November 2007, the estimated number of infected individuals worldwide was 33.2 million, with an incidence of 2.5 million [1]. Sixty-eight percent of these new cases occurred in sub-Saharan Africa. Over two million people died from AIDS in 2007 (Table 1). Since 2001, the number of infected individuals in Eastern Europe and Asia has increased by 150%. However, it has been reported that the overall prevalence of HIV has leveled off and the number of new infections has decreased [1].

In the twenty-five years since the discovery of the AIDS virus, the epidemic patterns have constantly evolved and the schematic maps continued to change colors. Though every year the numbers have increased, the world is beginning to see the pay off from all the efforts put forth to help eliminate the devastation caused from this disease. In countries in just about every region, there has been decreased prevalence in adults credited to signs of decreased risky behavior. Due to anti-retroviral therapy, HIV/AIDS mortality rate in many countries have declined in recent years [2]. Young women who attend antenatal have a lower prevalence that shows efforts are in fact beneficial in many of the most affected countries. Many countries of

Sub-Saharan Africa such as Kenya and Zimbabwe, though still the region with the highest number of infected individuals, show steadying or declining numbers.

Though success is apparent, much effort is still necessary. In some countries, there have been reversals from declining to increasing numbers of affected individuals. In areas like Latin America and the Middle East, there are existing stigmas and prejudices that inhibit proper surveillance and prevention and treatment efforts that need to be overcome [2]. With advances in this area, more accurate estimations will enable efforts tailored to the populations with most need.

**Table 1:** ***Current Estimations of the HIV/AIDS Epidemic by Region (2007).***

| World Region | Total People Living with HIV | New HIV Infections | Adult Prevalence | Total Deaths Due to AIDS |
|---|---|---|---|---|
| **Asia** | 4.9 Million | 440,000 | 0.1% | 300,000 |
| **Sub-Saharan Africa** | 22.5 Million | 1.7 Million | 5.0% | 1.6 Million |
| **Caribbean** | 230,000 | 17,000 | 1.0% | 11,000 |
| **Eastern Europe and Central Asia** | 1.6 Million | 150,000 | 0.9% | 55,000 |
| **North America and Western Europe** | 2.1 Million | 78,000 | 0.5% | 32,000 |
| **Latin America** | 1.6 Million | 100,000 | 0.5% | 58,000 |
| **Middle East and North Africa** | 380,000 | 35,000 | 0.3% | 25,000 |
| **Oceania** | 75,000 | 14,000 | 0.4% | 1,200 |
| **Total** | 33.2 Million | 2.5 Million | 0.8% | 2.1 Million |

# 2.0 CHAPTER TWO: BACKGROUND

## 2.1 HIV-1: VIRUS STRUCTURE AND PATHOGENESIS

The Human Immunodeficiency Virus type 1 (HIV-1), the causal agent of AIDS, is of the genus lentivirus and family *Retroviridae*. The virus contains two copies of its single-stranded RNA genome which encodes for structural and functional proteins as well as the promoter that allows for transcription of viral genes in host cells (Figure 1). This promoter is called the long-terminal repeat (LTR) and is flanked on each end of the coding region of the genome. Structural proteins include Gag, Pol, and Env which also are involved in the pathogenesis of HIV-1. Auxiliary or regulatory proteins comprise of Tat and Rev which are essential for viral replication. Nef, Vif, Vpr, and Vpu are accessory proteins, which while not necessary for viral reproduction, do aid in the efficient replication and pathogenesis of the virus.

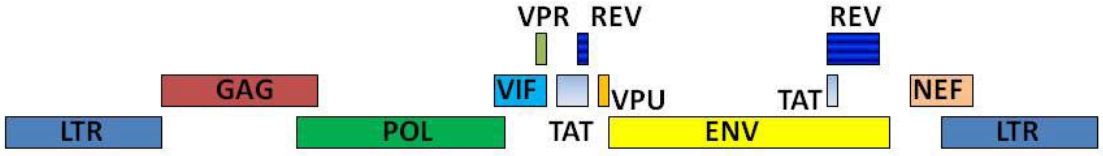



**Figure 1:** ***The HIV-1 Genome.***
**This schematic represents the HIV-1 genome including both structural and accessory proteins in which are flanked at either end by the HIV-1 LTR (long terminal repeat) promoter region. The structural proteins include *gag*, *pol*, and *env* while the remaining genes have various regulatory and accessory roles.**

Figure 2 depicts the general HIV-1 virus life cycle. Briefly, the virus attaches to the target cell surface via CD4 and the coreceptors, CCR5 or CXCR4, depending on the viral strain and target cell type, and the viral capsid is deposited into the cytoplasm [3, 4]. The viral RNA genome is reverse transcribed by virus encoded reverse transcriptase (RT) into double stranded DNA and translocalized to the nucleus where it integrates into the host genome.

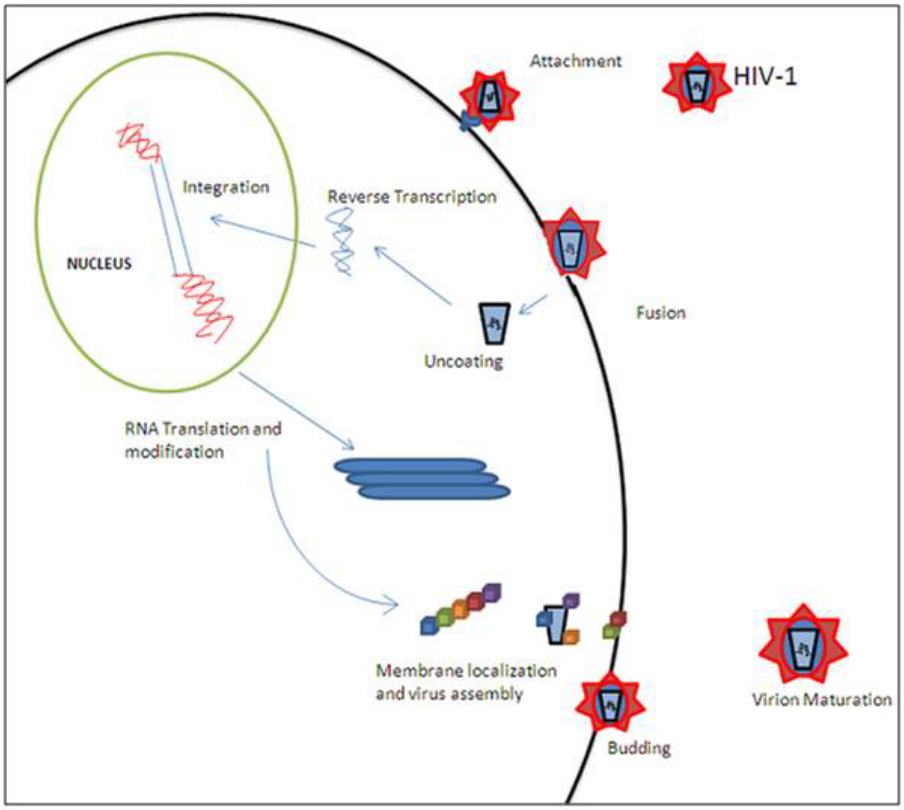



**Figure 2:** ***HIV-1 Viral Life Cycle.***
**This schematic represents the basic steps in HIV-1 infection at the cellular level.**

Transcription of proviral DNA by the host RNA polymerase II produces full-length transcripts that are used to produce viral proteins or genomic RNA. Env is translated in the endoplasmic reticulum and processed via the Golgi apparatus. Virion components assemble at budding sites at the interface of the cellular plasma membrane. Maturation continues after egress with protein

modification by virus encoded protease. The target cells for HIV-1 are dividing CD4+ T-cells and non-dividing cells of the macrophage-phagocytic system [3, 4]. These cells are functionally disrupted and/or depleted during the course of infection, disrupting the host immune system.

## 2.2 VIRAL PROTEIN R (VPR)

As previously mentioned Vpr is one of the four accessory proteins of HIV-1 and is the focus of this thesis. Vpr is described as a virion associated pleiotropic protein that has been associated with cell cycle arrest, apoptosis, immune regulation and evasion, translocation of the pre-integration complex (PIC) into the nucleus, and viral gene transactivation [5-8]. Viral protein R (Vpr) is a 14-kDa protein that consists of ninety six amino acids and structurally contains three α helical domains flanked by amino and carboxyl terminal ends (Figure 3) [7].

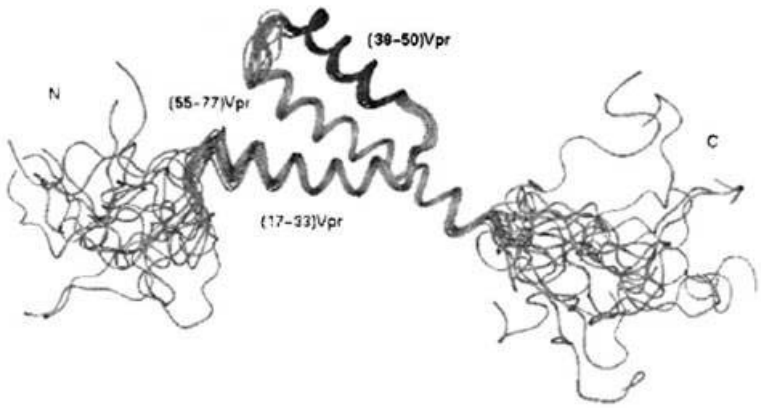



**Figure 3:** ***HIV-1 Vpr Protein Structure.***
**This schematic depicts Vpr's three alpha helices and the amino (N) terminal and carboxyl (C) terminal ends. Permission granted by Mary Ann Leibert, Inc. Publishers for use of this figure [7].**

These N-terminal and central regions have shown to play a role in the ability of Vpr to affect virion packaging, transcriptional activation and apoptosis [5, 7, 9]. The C-terminal region is arginine-rich and is also known to affect nuclear localization and the host cell cycle but it is not involved in virion incorporation [7, 9].

The focus of this thesis is on Vpr and its role in transcriptional regulation. Vpr has been specifically associated with activation of the HIV-1 LTR promoter, though modestly, and of several other heterologous promoters [7, 10]. Vpr is known to interact with transcription factors SP1 and TFIIB, as well as NFκB and AP-1 in primary macrophages, and the host cellular factors glucocorticoid receptor (GR) as well as progesterone and estrogen receptors [7, 11-13]. Vpr has the ability to accomplish these functions in multiple contexts. It has been shown that Vpr exists not only as virion or cell associated, but also as free Vpr (cell and virus-free) [14]. This thesis will cover the transcriptional regulation of host cytokine expression by Vpr in its various forms.

## 2.3 TUMOR NECROSIS FACTOR-ALPHA AND HIV-1 INFECTION

It has been proposed that immune dysregulation is an effect caused by HIV-1 infection and that cytokines play a large role. Tumor necrosis factor-alpha, referred to as TNF-α, is a proinflammatory cytokine secreted by cells of myeloid lineage and is known to be involved in inflammation and apoptosis. TNF-α is synthesized as a 233 amino acid structure which is cleaved, and trimerizes into its active state and acts extrinsically through its receptors TNFR1 and TNFR2 [13, 15, 16]. TNF-α matures and activates antigen presenting cells (APCs) as well as

other immune cells. Many studies have shown TNF-α and other inflammatory molecules to be associated with HIV-1 infection itself and also its associated anomalies such as lipodistrophy and AIDS-associated dementia. More specifically, recent studies have shown relationships between HIV pathogenesis, viral proteins and TNF-α signaling especially in the central nervous system and the brain.

It is generally accepted that TNF-α and TNFR levels are increased during the early phases of disease. These levels correlate with viral load and CXCR4 expression, and inversely correlate with $CD4^+$ T-cell counts [17, 18]. TNF-α produced by antigen-presenting cells (APCs) can cause secretion of other cytokines which affect infected and also bystander cells [19]. Interestingly, HIV-1 proteins including Nef, Vpr, and Tat have been proposed to act in a similar pathway as TNF-α, activating NFκB, AP-1 and mitogen-activated protein kinase (MAPK) to modulate cellular machinery [13, 18, 19]. This give strong evidence that TNF-α should be more closely studied in the context of HIV-1 infection.

## 2.4 DC IMMUNOLOGY AND HIV-1 INFECTION

During initial HIV-1 infection, especially when transmitted by sexual contact, it is understood that the first interaction between immune cells and virus occurs in the genital and oral mucosal surfaces [20]. Since dendritic cells (DCs) are located here, they are proposed to be one of the first cell types to encounter HIV. Though DCs express low levels of CCR5 and CXCR4 receptors, they have the ability to uptake virus due to their endocytotic characteristic as well as the expression of multiple C-type lectins such as DC-SIGN that are able to bind to HIV Env [21].

DCs travel into the lymphoid tissues where they act as antigen presenting cells to $CD4^+$ and $CD8^+$ T cells [20]. This is a proposed route of virus spread to the localized T-cell populations [20, 22]. HIV-1 is able to modulate normal DC function in infected individuals. For example, infected DCs express lower levels of surface co-stimulatory molecules such as CD80 and CD86 and are significantly less efficient at inducing DC dependent T cell responses [20].

HIV-1 Vpr is known to modulate normal DC phenotype and function as well as differentially regulate the expression of TNF-α. HIV-1 replication in DCs occurs very inefficiently. However, cellular factors such as cytokines including TNF-α are able to influence viral replication in macrophages, DCs, and T cells. Dendritic cells play a large role in the induction of a proinflammatory response by activation of cytokine signaling cascades. However, there is a constant battle between proinflammatory molecules which aids in viral replication due to enhanced immune cell migration, and the viral suppressive activity of other cytokines [23]. For example, TNF-α enhances HIV-1 replication in macrophages and DCs as well trigger apoptosis of bystander T cell populations; however IL-15 is a potent inhibitor of the TNF-α apoptosis pathway [18, 19, 24]. IL-15 production is impaired during HIV-1 infection specifically in the presence of Vpr in DCs [24, 25]. Therefore, Vpr differentially regulates multiple cellular factors that can modulate normal immune function and the mechanisms involved are of importance for further understanding of HIV-1 infection.

# 3.0 CHAPTER THREE: THESIS AIMS

HIV-1 Vpr differentially regulates cytokine expression during HIV-1 infection causing alteration of normal immune cell function. Several viral proteins, including Vpr are implicated in this immune modulation induced by HIV-1. Proinflammatory cytokine TNF-α is known to be regulated during early infection. We hypothesize those viral proteins that are a part of the virus particle, particularly HIV-1 Vpr, might have an impact on TNF-α production.

**AIM #1: To investigate the effects of HIV-1 Vpr on the TNF-α production by dendritic cells.**

A. Explore the effects of Vpr in its various forms on TNF-α production
B. To map the domains of Vpr responsible for the induction of TNF-α in dendritic cells
C. Determine whether infected cells and/or uninfected bystander cells are responsible for the increased TNF-α expression by Vpr

Vpr is known to transactivate viral and host cellular promoters as well as act as a co-activator of viral andgene expression during infection. We hypothesize that regulation of TNF-α expression by HIV-1 Vpr might be mediated at the TNF-α promoter level.

**AIM #2: To determine the domains of the TNF-α promoter involved in the transactivation of TNF-α expression by HIV-1 Vpr.**

# 4.0 CHAPTER FOUR: MATERIALS AND METHODS

## 4.1 CELL LINES

HEK293T, 293FT, microglia, and HeLa T4 cells were maintained in DMEM (GIBCO) supplemented with 10% FBS, 1% L-glutamine (Cambrex), 1% penicillin-streptomycin (GIBCO), and 1% 10mM Non-Essential Amnio Acids (GIBCO); medium for 293FT additionally contained 500µl/ml Geneticin. 293FT cells were purchased from Invitrogen as a component of the pLenti/V5 Directional TOPO Cloning Kit. HEK293T cells were given by Dr. Michelle Calos, Stanford University, CA. HeLa T4 cells were from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, UNAID, NIH contributed by Dr. Richard Axel [26]. Microglia cells were provided by Dr. Bassel Sawaya at Temple University, Philadelphia, PA.

Monocyte-derived dendritic cells (DC) were derived from human peripheral blood mononuclear cells (PBMC) from heparinized blood obtained from anonymous healthy, HIV-1 negative, donors by the Central Blood Bank of Pittsburgh, PA with appropriate IRB approval. PBMC were isolated by Fycoll-Hypague gradient centrifugation. From these cells, $CD14^+$ monocytes were purified by positive selection using anti-CD14 monoclonal antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as the protocol suggests. Briefly, to obtain

monocyte-derived DC, $CD14^+$ cells ($0.5X10^6$ cells/ml) were cultured in 60 mm culture plates in a total volume of 6 mL RPMI (GIBCO, Gaithersburg, MD) media containing 10% FBS, 1% L-glutamine (Cambrex, Walkersville, MD), 1% penicillin-streptomycin (GIBCO), 25 ng/ml IL-4 (R&D Systems, Minneapolis, MN) and 50 ng/ml recombinant GM-CSF (R&D Systems). Half the volume of media was replaced every second or third day with fresh media containing IL-4 and GM-CSF throughout the entire culture period.

## 4.2 CLONING

The Vpr-EGFP lentiviral plasmid was cloned using the pLenti/V5 Directional TOPO Cloning Kit (Invitrogen). The Vpr-EGFP fusion gene, previously constructed, was amplified by PCR using forward primer: 5'CACCATGGAACAAGCCCCAGAGA3' and reverse primer: 5'TTACTTGTACAGCTCGTCCAT3' producing a blunt-end product for proper fusion to the V5 epitope flag of the cloning vector. The PCR product was transformed into One Shot® Stbl3™ competent cells, cultured in S.O.C. media for one hour, then plated on LB agar plates containing ampicillin (100μg/ml). Single colonies were cultured in ampicillin containing media and DNA was screened by restriction digest analysis for positive clones. The Vpr lentivirus plasmid was obtained by restriction digest of Vpr-EGEF lentivirus plasmid removing the EGFP fragment with Age I. The EGFP lentivirus was obtained similarly.

## 4.3 TRANSFECTION METHODS

### 4.3.1 Calcium Phosphate

The calcium phosphate method was used for DNA transfection of HEK293T cell line. Briefly, cells were cultured in 10mm dishes to approximately 75% confluency. Four hours prior to transfection, old media was replaced with 6 ml DMEM supplemented with 10% FBS and cells were placed in 5% $CO_2$. The transfection was conducted by adding DNA (up to 25µg) to 450µl water, then subsequently adding $CaCl_2$ (50µl) and 50mM BES (BES, 250mM NaCl, 0.5mM $Na_2HPO_4$) (450µl). The transfection mixture was allowed to incubate at room temperature for 30 minutes. The mixture was then added drop-wise to the cell media. Twelve to 16 hours later, the cell media was removed and replaced with fresh complete media.

### 4.3.2 Lipid-Mediated Transfection

Lipid-mediated transfection method was performed using 293FT, HeLa T4, and Microglia cell lines. Cells were plated in antibiotic-free media for approximately twelve hours prior to transfection to 90% confluency. For a 12-well plate, DNA (1-2µg) was added to Opti-MEM (GIBCO) (50µl). Lipofectamine 2000 transfection reagent (Invitrogen) (1µl) was added to Opti-MEM (50µl) and incubated at room temperature for 5 minutes, then added to the DNA mixture. The DNA-Lipofectamine complex was incubated at room temperature for 20 minutes. Post-incubation, cell culture media was replaced with fresh antibiotic-free DMEM and the transfection mixture was added drop-wise to the culture. The cells were incubated at 37ºC for 4-

6 hours, then the medium was replaced with fresh DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 1% 10mM Non-Essential Amino Acids.

## 4.4 VIRUS

### 4.4.1 Virus Production

HIV-1 NL43 and 89.6 wild-type and ΔVPR viruses were obtained by transfection of HEK293T cells with pNL43 or 89.6 WT or ΔVPR proviral plasmids by calcium phosphate method. Virus released in the supernatant was collected seventy-two hours post transfection. Lentiviruses were produced by lipid-mediated transfection of the specified lentiviral plasmid accompanied by the Virapower® Packaging Mix (Rev, Gag/pol, VSV/G) in the 293FT cell line. All viruses were purified by centrifugation and filtration to remove cellular debris, and concentrated by ultracentrifugation at 22,000 rpm for 1 hour at 4ºC. Viral titer was determined through p24 antigen ELISA.

### 4.4.2 Virus Labeling

NL43 wild-type and NL43 ΔVPR viruses were labeled directly with Cy5 mono-reactive dye as suggested by the manufacturer's protocol (Amersham). Initial protein estimations of the virus samples were taken by Bradford protein assay. One milligram of total virus protein was dissolved in 1mL of 0.1M sodium carbonate buffer and then added to one vial of dye. This was mixed thoroughly by inverting the vial, and then incubated at room temperature mixing every ten

minutes. Labeled virus was separated from free dye through the use of a G-25 M Sephadex column (Amersham). Column was equilibrated with DPBS twice. Reaction mix was added to the column and further DPBS was added to allow flow through. Two bands were seen, the faster moving band was labeled virus, while the slower moving band was the free dye. Virus titer was measured by p24 antigen ELISA post-labeling.

## 4.5 WESTERN BLOT

For virus characterization, HEK293T and 293FT cells were derived from untransfected and transfected cells and virus pellet was obtained as described above. Cells were washed twice with PBS and lysed in RIPA buffer containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton-X100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1.0mM phenylmethyl-sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin inhibitor units/ml aprotinin, and 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 SDS-PAGE gel, transferred to a membrane, and immunoblotted for with anti-HIV-1 p24 (NIH AIDS Research and Reference Reagent Program), anti-HIV-1 Vpr (gift from John Kappes, University of Alabama), and/or anti-GFP (Abcam) for detection of mentioned proteins. Loading was based on protein concentration.

Western Blots were also done on virus pellets to confirm the concentration of the p24 gag protein. Vpr was also detected in HIV-1 Vpr(+) virus pellets. Pellets were separated on a SDS-PAGE gel, transferred to a membrane, and the expression of the above mentioned proteins were detected as described before.

## 4.6 VPR PEPTIDES

VPR peptides used in DC stimulation experiments were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus B VPR (15-mer) Peptides - Complete Set. Sets used in these specific experiments were made by combining various 15-mer peptides to obtain desired sequence (Table 2).

**Table 2:** ***HIV-1 VPR Peptides.***

| | **Peptide Sequence** |
|---|---|
| **1-19** | MEQAPEDQGPQREPHNEWT |
| **21-35** | ELLEELKNEAVRHFP |
| **37-55** | IWLHGLGQHIYETYGDTWA |
| **45-63** | HIYETYGDTWAGVEAIIRI |
| **65-83** | QQLLFIHFRIGCRHSRIGV |
| **81-96** | IGVTRQRRARNGASRS |

DCs were isolated and cultured as previously described. Four days after isolation, DCs were exposed to different Vpr peptides (100ng/ml). Forty eight hours after stimulation, LPS was added or not added to the cell culture medium. Cell supernatants were collected after an additional 24 hours and stored at -80ºC until analysis by TNF-α ELISA as described below. Values were compared to TNF-alpha production from stimulation with irrelevant Vif peptides obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (HIV-1 Consensus B VIF (15-mer) Peptides - Complete Set).

## 4.7 RECOMBINANT PROTEIN

Vpr recombinant protein was produced using the pET Duet vector and protein expression system (Novagen). Glutathione *S*-transferase (GST) recombinant protein was used as an irrelevant control protein. Both Vpr and GST were produced in *E. coli* following IPTG induction and purified according to the manufacturer's instructions (Novagen). Bacterial contaminants were removed by high-performance liquid chromatography (HPLC) purification and absence of endotoxin was confirmed as described by our laboratory previously [19].

DCs were cultured as described before. Recombinant Vpr or GST protein was added to the cells in various concentrations and incubated for 48 hours. Cells were then treated with or without LPS for an additional 24 hours. Supernatants were collected and used for cytokine analysis by TNF-α ELISA as described below.

## 4.8 CYTOKINE ANALYSIS OF HIV-1 EXPOSED DC

### 4.8.1 Detection of TNF-alpha by ELISA

Dendritic cells were isolated and cultured as previously described. Four days after initial culture, DCs were exposed to HIV-1 wild-type or HIV-1 ΔVPR. After forty-eight hours post-transduction, cells were stimulated with LPS (1μg/ml). Twenty-four hours post LPS stimulation, supernatants and cells were collected for further analysis. Cell debris was removed from the

supernatants by centrifugation. Soluble TNF-α was measured in these supernatants by TNF ELISA Set as per manufacturer's instructions (BD Biosciences).

### 4.8.2 Flow cytometry

DCs were treated as mentioned above. For flow cytometry, cells were collected and washed two times with FACS buffer (PBS supplemented with 3% FBS). For surface staining to detect expression of IL-15, cells were stained with IL-15 antibody (Santa Cruz Biotechnologies) for 30 minutes on ice. Cells were washed and conjugated with anti-$IgG_1$-PE (Santa Cruz Biotechnologies) also on ice for 30 minutes. Cells were washed twice with FACS buffer to remove residual antibody. Cells were fixed in 2% paraformaldehyde at 4ºC.

For intracellular detection of TNF-α and/or p24, virus exposed cells were treated with GolgiStop® (BD Biosciences) in addition to LPS as described above. This inhibits anterograde transport of cytokines from the golgi apparatus to the cell surface. Cells were collected and washed two times with FACS buffer. For detection of intracellular proteins, fixation and permeabilization was carried out using the Cytofix/Cytoperm kit (BD Bioscience). The cells were incubated in the Cytofix/Cytoperm solution for 20 minutes at room temperature, followed by two washes with the Perm/Wash buffer (BD Bioscience). Intracellular staining was performed at room temperature for one hour then washed with Perm/Wash and analyzed by flow cytometry in FACS buffer. Antibodies used include anti-mouse TNF-PE (BD), anti-rat TNF-APC (Abcam), and for p24 detection anti-K562-FITC (Beckman Coulter). Analysis was done using the computer program, FlowJo.

## 4.9 TNF PROMOTER-LUCIFERASE ASSAY

### 4.9.1 Promoter-Luciferase Constructs

TNF-luciferase constructs were kindly provided by Dr. Bassel Sawaya, Temple University, Philadelphia, PA. Constructs were confirmed by enzyme digest and sequencing reactions.

### 4.9.2 Luciferase Reporter Assay

Cells were cultured in a 12-well plate format. TNF-α promoter luciferase constructs (1μg) were transfected using the lipid-mediated transfection method mentioned above. In microglia cells, pVPR or pcVector (pcDNA3.1/V5 HIS TOPO Expression Vector, Invitrogen), was co-transfected with the luciferase construct. Twenty-four hours post-transfection, LPS (1μg) was added to the cells. In HeLa T4 cells, transfection of the TNF-α promoter-luciferase constructs was performed by lipid-mediated transfection. HIV-1 NL43 wild-type or ΔVPR (100ng p24 equivalent per ml) was added in addition to PMA (20ng) and ionomycin (5μM) 24-hours post-transfection. The assay was performed using the Luciferase Assay System (Promega) as the protocol suggested. Briefly, forty-eight hours post-transfection, culture medium was removed and discarded. Cells were washed with PBS and all liquid was removed. Passive Lysis Buffer (200μl of 1X) was added to the cells and cell lysate was separated from cell debris by centrifugation. Cell lysates (20μl) were added to Reporter Assay Substrate (100μl) and relative light units were detected by luminometer. Transfection efficiency was normalized by co-transfecting pEGFP with the promoter-luciferase constructs and determining equivalency by fluorescence microscope.

## 4.10 STATISTICAL ANALYSIS

Results were analyzed using 1-tailed, paired student t-test using Microsoft Excel. P value less than $\leq 0.05$ was considered as statistically significant.

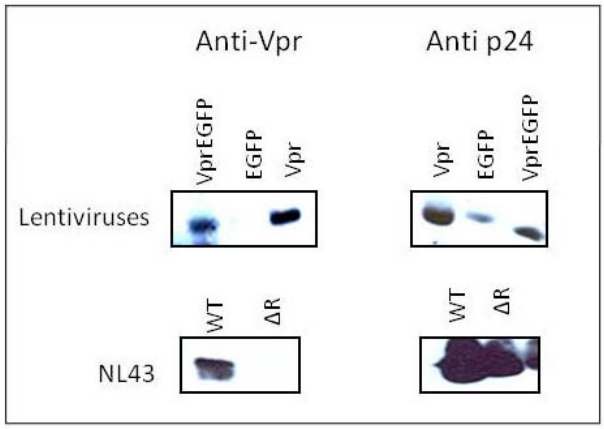
# 5.0 CHAPTER FIVE: RESULTS

## 5.1 AIM #1: TO INVESTIGATE THE EFFECTS OF HIV-1 VPR ON THE TNF-ALPHA PRODUCTION BY DENDRITIC CELLS

### 5.1.1 Characterization of Viruses

To identify the effects of HIV-1 Vpr multiple virus constructs were used. First, HIV-1 NL43 (CXCR4-tropic) and 89.6 (dual tropic) viruses, and lentiviruses expressing Vpr or control protein (EGFP) were generated as described in the above section. Characterization was completed for the presence of p24 Gag antigen as well as for Vpr using specific antibodies. As seen in Figure 4A, p24 Gag was detected by protein analysis of concentrated virus pellet by western blot. Presence or absence of Vpr was also determined by western blot. As expected, viruses containing Vpr mutations had no trace of the protein. p24 Gag was also detected in virus exposed dendritic cells by flow cytometry confirming the occurrence and efficiency of virus transduction (Figure 4B). Equal to reduced transduction was typically seen by HIV-1 Vpr(-) virus compared to Vpr-containing wild type virus.

A.




B.

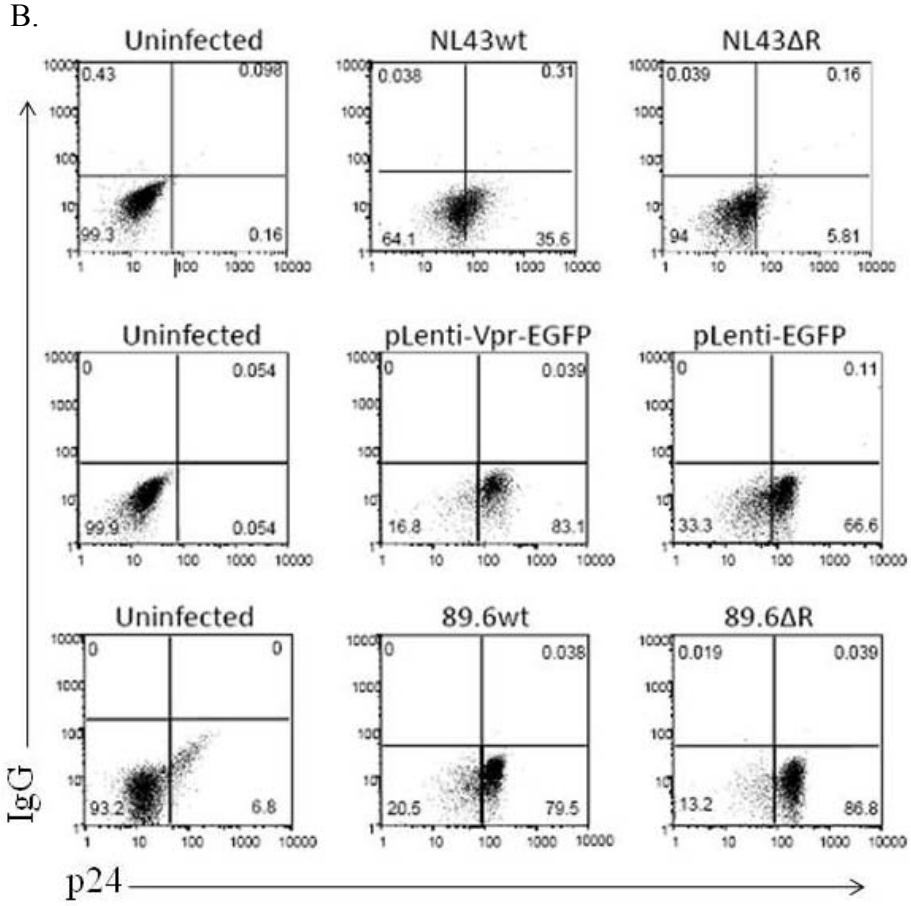



**Figure 4:** ***Viral protein Characterization of Viruses.***
**(A)Virus pellets analyzed for the presence or absence of p24 Gag and Vpr by western blot, (B) Virus transduction in exposed DCs determined by flow cytometry after intracellular staining for Gag p24 antigen.**

Lentiviruses expressing EGFP were further characterized by fluorescence microscopy in 293FT cells during virus production (Figure 5). Expected patterns were observed as the pLenti-EGFP transfected cells showed protein expression localized in the cytoplasm. Cells transfected with pLenti-Vpr-EGFP were seen with expression in both the cytoplasm and the nucleus. This would occur due to Vpr nuclear localization with the fusion protein remaining intact.

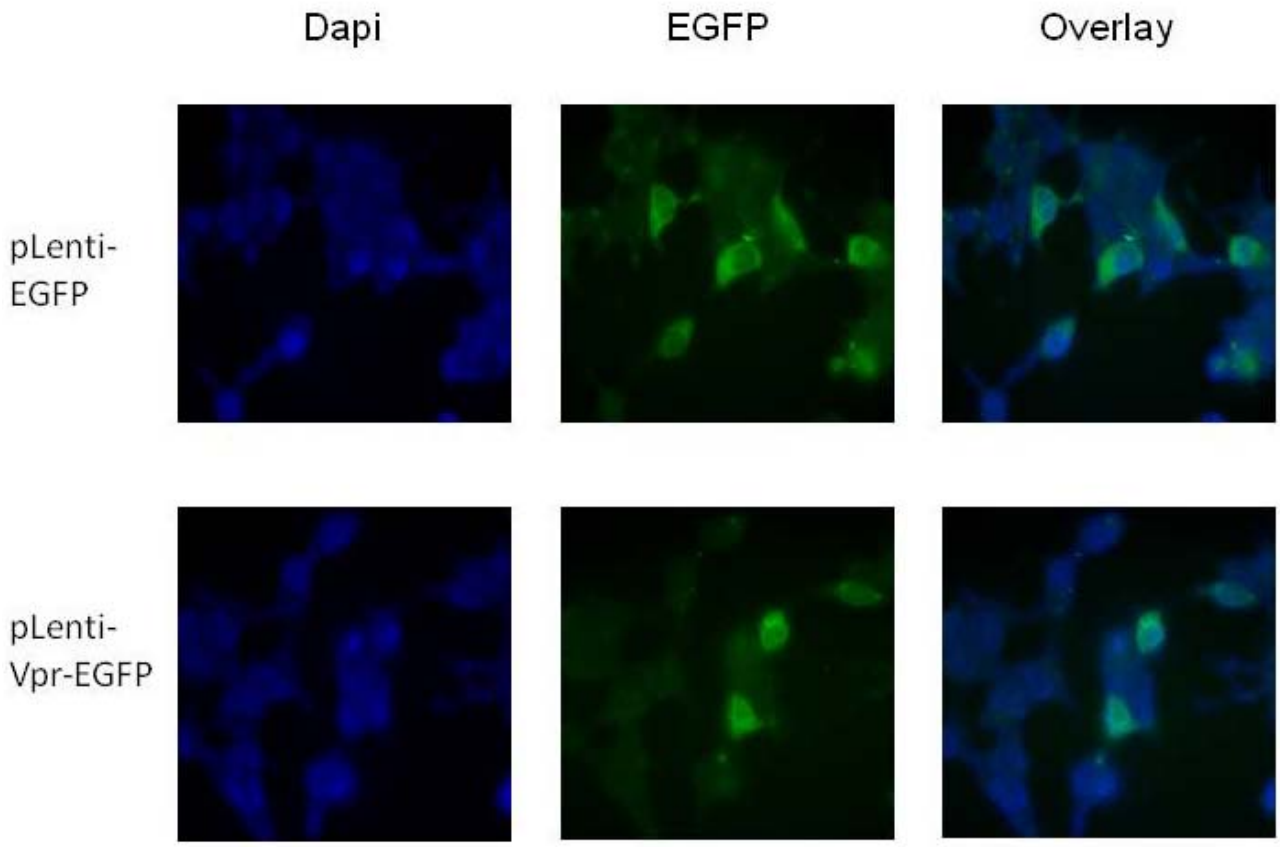



**Figure 5:** ***Characterization of Vpr-EGFP expressing lentivirus constructs by immunofluorescence.***
**Characterization of lentiviral constructs was performed by transfecting 293FT cells with the appropriate plasmid, followed by fluorescent microscopy. The cell nucleus was stained with Dapi stain (blue) and EGFP expression (green) was observed using FITC filters. Localization of protein expression can be seen with the overlay.**

### 5.1.2 Induction of TNF-α by HIV-1 Vpr

Vpr is known to play a role in activation of viral protein expression and signaling cascades that elicit proviral and antiviral responses. In dendritic cells, our laboratory has shown that HIV-1 Vpr(+) virus exposure causes an upregulation of TNF-α mRNA synthesis and protein expression [19]. Similar effects were observed in DCs exposed to noninfectious virus containing Vpr as well as Vpr-GST recombinant protein. This section reiterates previous findings and gives further insight to Vpr's role in TNF-α production by dendritic cells in regards to its multiple forms and protein domains.

#### 5.1.2.1 Affect on TNF-α by Vpr in the Context of HIV-1 Infection

To assess Vpr and its ability to induce TNF-α expression in the context of infection, DCs were cultured as described and exposed to VSV-G pseudotyped HIV-1 NL43 wild-type or Vpr defective virus (Figure 6). VSV-G enables the NL43 variant to be taken up by dendritic cells due to the X4 tropism of the viral strain and low expression of the CXCR4 co-receptor on DC surfaces. TNF-α was detected in the DC supernatant by ELISA and intracellularly by flow cytometry. There was an average two fold increase in TNF-α production by NL43 Vpr(+) virus infected DCs upon LPS stimulation. Similar results were observed in six separate donors. A similar increase in TNF-α expression was also detected by flow cytometry with over twenty percent more in cells exposed to Vpr(+) virus.

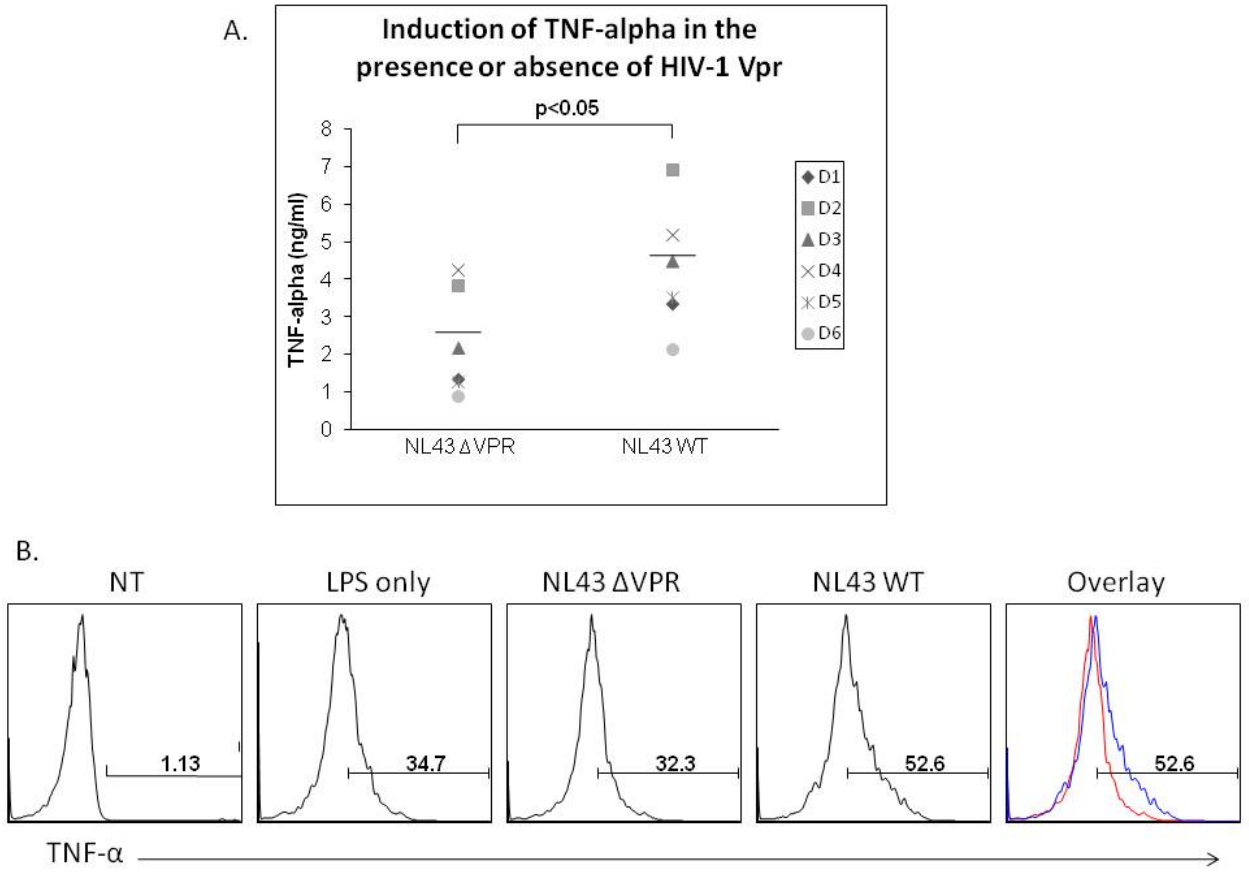



**Figure 6:** ***Increase in TNF-alpha expression by HIV-1 Vpr in the context of virus infection.*** **(A) DCs were exposed to NL43 Vpr(+) or NL43 Vpr(-) virus for 48 hours then stimulated with LPS for an additional 24 hours. Supernatants were then collected for analysis by TNF-α ELISA. Solid lines indicate donor averages. (B) DCs were exposed to NL43 Vpr(+) or NL43 Vpr (-) for 48 hours then stimulated with LPS in the presence of GolgiStop for 6 hours. Cells were permeablized for intracellular staining of TNF-alpha and expression was detected by FACS analysis (representative of six donors).**

Similar experiments were done next using HIV-1 89.6 wild-type and 89.6 ΔVpr viruses (Figure 7). HIV-1 89.6 viral strain is CCR5/CXCR4 dual-tropic and have the ability to infect macrophages without VSV-G, however to keep consistency between experiments, these viruses were pseudotyped as well. After virus transduction of DC and stimulation with LPS as previously mentioned, TNF-α was measured intracellularly or by secretion in the cell

supernatants. On average, there was a 2.7 fold increase in TNF-α secretion by Vpr containing virus compared to in counterpart, which was statistically significant. Minimal two-fold differences in TNF-α expression were also consistently seen by intracellular staining. Together, these results indicate a similar induction of TNF-α expression by Vpr independent of the HIV-1 viral strains used to transduce DCs.

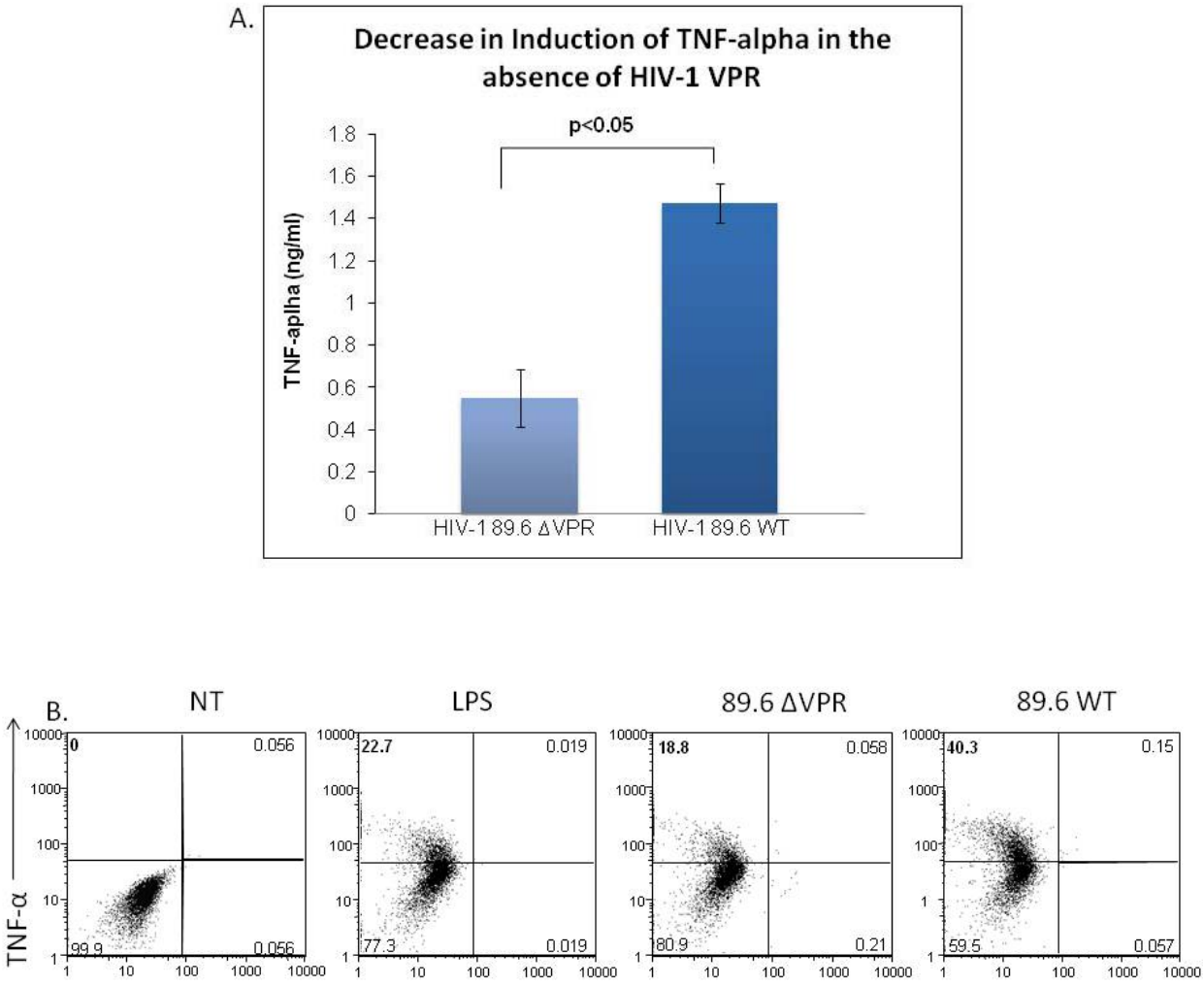



**Figure 7:** ***Increase in TNF-alpha expression by HIV-1 Vpr in the context of 89.6 infection.*** **(A) DCs were exposed to 89.6 Vpr(+) or 89.6 Vpr(-) for 48 hours then stimulated with LPS for an additional 24 hours. Supernatants were then collected for analysis by TNF-α ELISA. Results include standard error across two separate donors. (B) DCs were exposed to 89.6 Vpr (+) or 89.6 Vpr (-) for 48 hours then stimulated with LPS in the presence of GolgiStop for 6 hours. Cells were permeablized for intracellular staining of TNF-alpha and expression was detected by FACS analysis (representative of two donors).**

To control for specificity of this effect seen on TNF-α, production of IL-15 by DCs was also detected by flow cytometry. In this case, an opposite effect on IL-15 expression on the DC cell surface was observed by the same donors used for the previous experiments as shown in Figure 8. As expected, induction of IL-15 expression in the presence of HIV-1 Vpr was decreased in comparison to Vpr defective virus. Similar results were observed when using either virus construct. This indicated that the increase in TNF-α expression by HIV-1 Vpr is a specific effect.

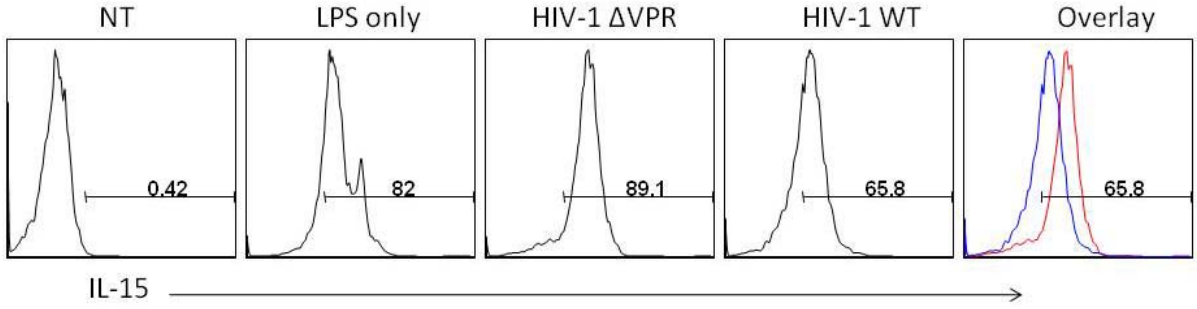



**Figure 8:** ***Effect of HIV-1 Vpr IL-15 expression.***
**DCs were obtained as described previously and exposed to HIV-1 Vpr(+) or HIV-1 Vpr(-) virus for 48 hours then stimulated with LPS for an additional 24 hours. Cells were stained for surface IL-15, fixed and analyzed by flow cytometry.**

### 5.1.2.2 Affect on TNF-α by *de novo* synthesized Vpr

Next, to assess the role of *de novo* synthesized HIV-1 Vpr and its affect on the production of TNF-α in DCs, cells were infected with lentiviruses expressing Vpr alone, the Vpr-EGFP fusion protein or EGFP alone, which was used as a control. By using a lentiviral construct, the encoded viral genome has the ability to integrate into the infected cell's genome and be expressed in this fashion. For initial infection ability, virus constructs were packaged with Gag, Rev, and VSV-G Env. As Figure 9 displays, *de novo* synthesized Vpr increased the production of

TNF-α as compared to EGFP alone. Vpr expressed as a fusion with EGFP induced an average 1.6 fold increase in TNF-α secretion by DCs where as Vpr alone induced a significant 3.5 fold difference compared to EGFP alone (Figure 9A). An increase was also seen by intracellular staining for TNF-α (Figure 9B). This gives insight to the difference *de novo* synthesis of viral proteins can make on the pathogenesis of HIV-1.

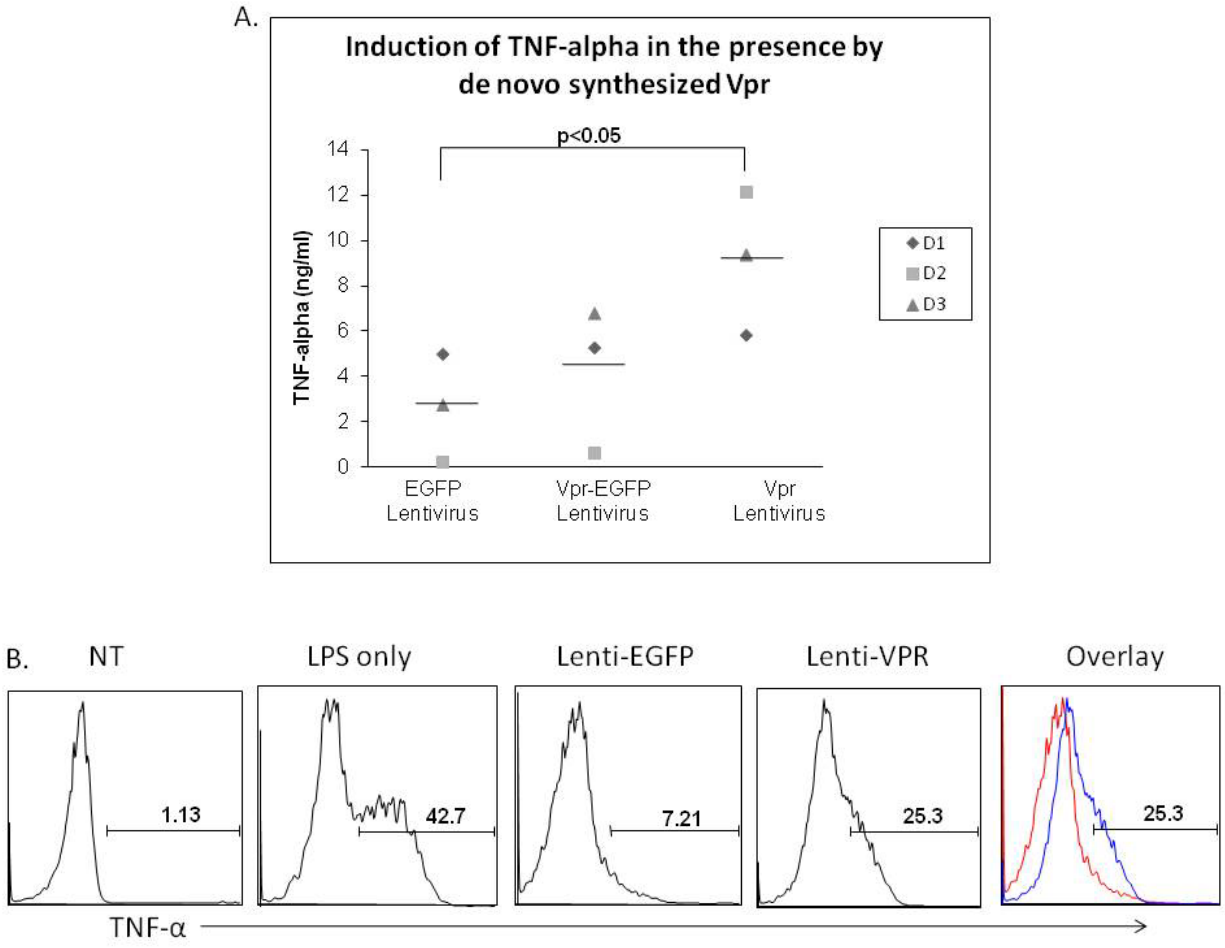



**Figure 9:** ***Increase in TNF-alpha production by de novo synthesized HIV-1 Vpr.***
**(A) DCs were infected with Vpr and/or EGFP containing lentiviruses for 48 hours and additionally with LPS for 24 hours. After stimulation supernatants were collected for TNF-α quantification by ELISA. (B) DCs were infected with Vpr or EGFP containing lentiviruses for 48 hours and stimulated with LPS in the presence of GolgiStop for 6 hours. Cells were permeablized and quantified for TNF-α by intracellular staining and FACS analysis (representative of three separate donors).**

### 5.1.2.3 Effect on TNF-α Expression by Recombinant Vpr Protein

To evaluate the effect of TNF-α expression by cell and virion free Vpr, DCs from three separate donors were exposed to multiple concentrations of recombinant Vpr and GST proteins in culture (Figure 10). As the concentrations increased, ultimately so did the level of TNF-α released by the DCs exposed to Vpr whereas the TNF-α secretions remained minimal when exposed to GST protein. Interestingly, there was a significant jump between exposure to 1µg/ml and 10µg/ml free Vpr which then tapered off up to 50µg/ml. Notably, the values measured for TNF-α after Vpr protein exposure were greatly increased compared to Vpr in the context of infection or de novo protein synthesis indicating a strong role of free Vpr during infection.

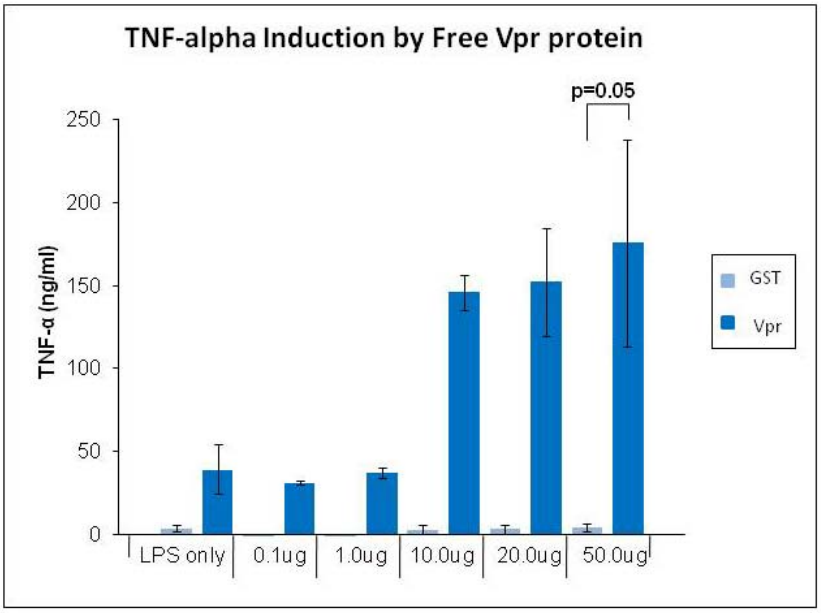



**Figure 10:** ***Increase in TNF-alpha production by recombinant Vpr protein.***
**DCs were cultured as described and exposed to Vpr or GST recombinant protein for 48 hours and stimulated with LPS for an additional 24 hours in 1ml of culture. Supernatants were collected and analyzed by TNF-α ELISA. Results include standard error between three separate donors.**

### 5.1.2.4 Domains of Vpr Involved in TNF-α Production

Next, to identify the domains of Vpr involved in the induction of TNF-α, human DCs were exposed to different Vpr peptides (15-mers). TNF-α was measured in the cell supernatants. TNF-α production was compared to that of irrelevant Vif peptides. Between the Vpr peptide groups, slight fluctuations were seen but no significant difference was measured (Figure 11). Results were consistent among five separate donors. The six domains studied show each set are equally involved in the ability of Vpr to induce the production of TNF-alpha.

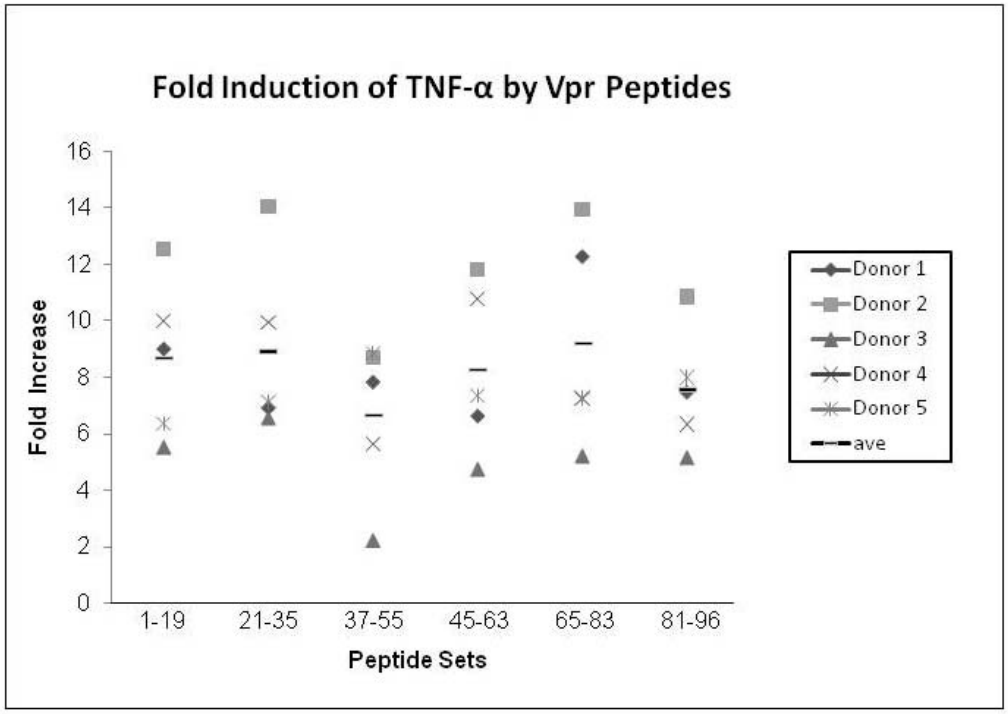



**Figure 11:** ***TNF-alpha production by Vpr Peptides.***
**DCs were exposed to 100µg of the indicated peptides for 48 hours. Cells were stimulated with LPS and supernatants were collected after 24 hours. Fold induction calculated by TNF production induced from 100ug Vpr peptides compared to production from irrelevant Vif peptides.**

### 5.1.3 Differentiation of DC Populations Responsible for Enhanced TNF-α Production

#### 5.1.3.1 Intracellular staining for p24 and TNF-α

It has been established that HIV-1 infection can cause inflammatory affects in those specific cells or in uninfected bystander cells. Using DCs from the same donors as previous experiments, it was sought out to distinguish whether the induction of TNF-α expression by Vpr occurred in infected cells or bystander cells. Cells were transduced with virus and stimulated with LPS then stained intracellularly for TNF-α or p24 Gag antigen, or both, and analyzed by flow cytometry (Figure 12). In Figure 12A, the top panel shows that TNF-α production was detected when stimulated cells were singly stained. The middle panel shows a shift of p24 positive cells when exposed to virus, however, in those only exposed to LPS as well. When stained for both markers, a similar pattern is seen in the uninfected cells with a p24 positive shift indicating nonspecific binding of the p24 antibody. This was seen in multiple donors, and without an alternative p24 antibody available, the same results were revealed after changing other assay parameters (Figure 12B). With this nonspecific p24 antibody staining, no conclusion could be drawn as to the percentages of infected/exposed or uninfected cells, nor which cells were the dominant producers of TNF-α.

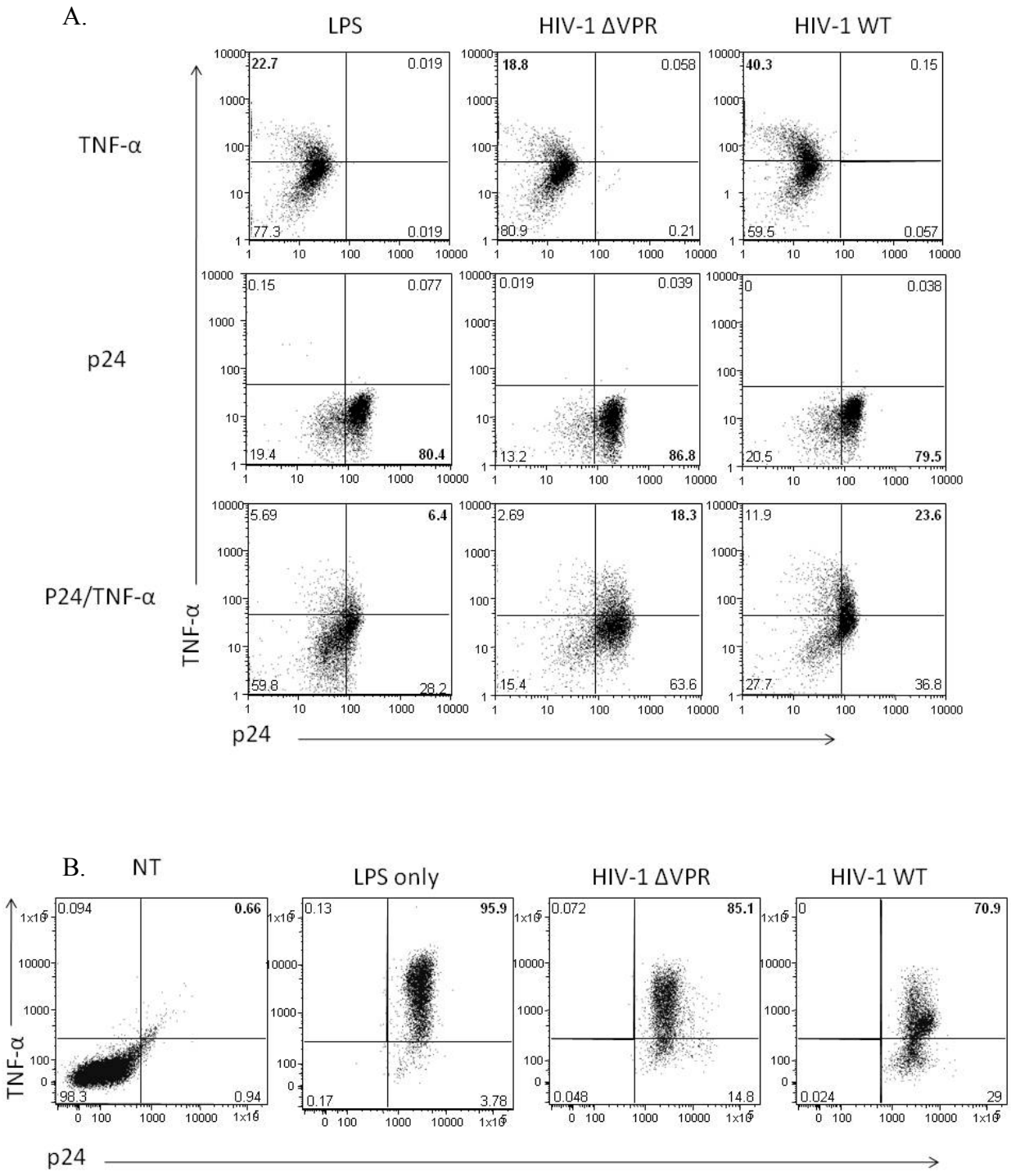



**Figure 12:** ***Dual staining of HIV-1 virus exposed DCs for TNF-alpha and p24 Gag.***
**DCs were cultured and exposed to HIV-1 89.6 WT or HIV-1 89.6 ΔVpr for 48 hour then exposed to LPS in the presence of GolgiStop for six hours. Cells were permeablized and stained for (A) TNF-α, p24 or (A and B) both and analyzed by flow cytometry.**

### 5.1.3.2 Cy5 labeled NL43 virus

To attempt to distinguish cell type specificity for the enhanced production of TNF-α in virus exposed DCs, a second methodology was used. VSV-G complemented NL43 Vpr(+) and NL43 (Vpr-) viruses were labeled using Cy5 dye as described in the Materials and Methods section. Cy5 can be detected by the APC channel of the cytometer and therefore can signify infected cells. Cy5-labeled virus was quantified by total protein due to p24 Gag antigen levels being low to undetectable after labeling by p24 ELISA. After virus exposure and LPS stimulation, cells were stained intracellularly for TNF-α and analyzed by flow cytometry.

First, to determine the ability of DCs to uptake this virus, multiple concentrations of the virus were added to the cells. Shown in Figure 13, as the added virus concentration increased, the virus uptake also increased reaching close to 100% Cy5+ positive cells, indicating the ability of the cells to uptake this virus and do so in a dose dependent manner.

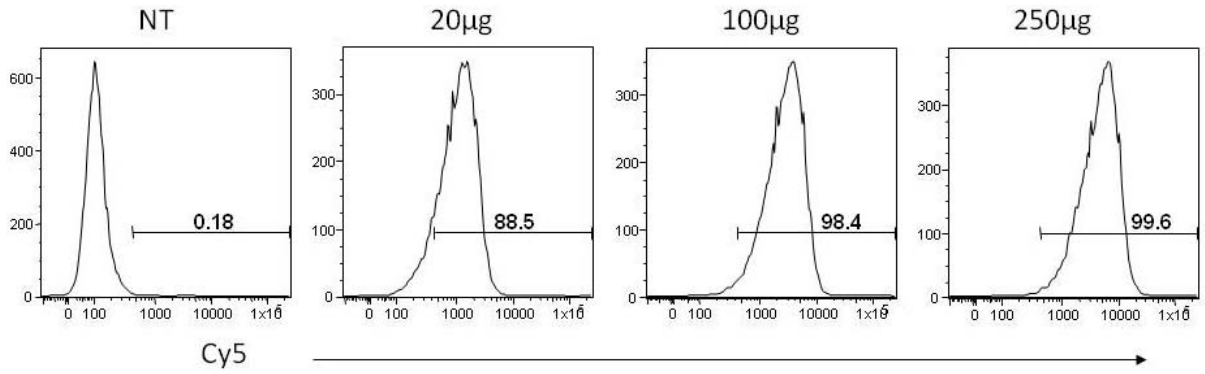



**Figure 13:** ***DC uptake and TNF-α production by Cy-5 labeled virus.***
**DCs were cultured as described and exposed to Cy-5 labeled HIV-1 WT or ΔVpr for 48 hours and stimulated with LPS in the presence of GolgiStop for 6 hours. Cells were harvested, permeablized and stained intracellularly for TNF-α and analyzed by flow cytometry (representative of at least three separate donors).**

Next, to assess the TNF-α production of DCs after Cy5-labeled virus exposure, the secretion of this cytokine was detected by ELISA (Figure 14A). At each concentration, both HIV-1 Vpr(+) and HIV-1 Vpr(-) virus exposure induced the expression of TNF-α at similar levels as non-labeled virus shown previously. Interestingly, a large difference between the two viruses was only seen when exposed to a higher virus dose. This is likely due to the increased concentration of p24 Gag present in this higher dosage. Due to this, 250ng was used in further experiments where DCs were stained for TNF-α for analysis by flow cytometry. As seen in Figure 14B, TNF-α was detected only in the double positive population demonstrating that responsible DC population is virus infected/exposed. This shows that virus exposed cells might be the primary producers of TNF-α.

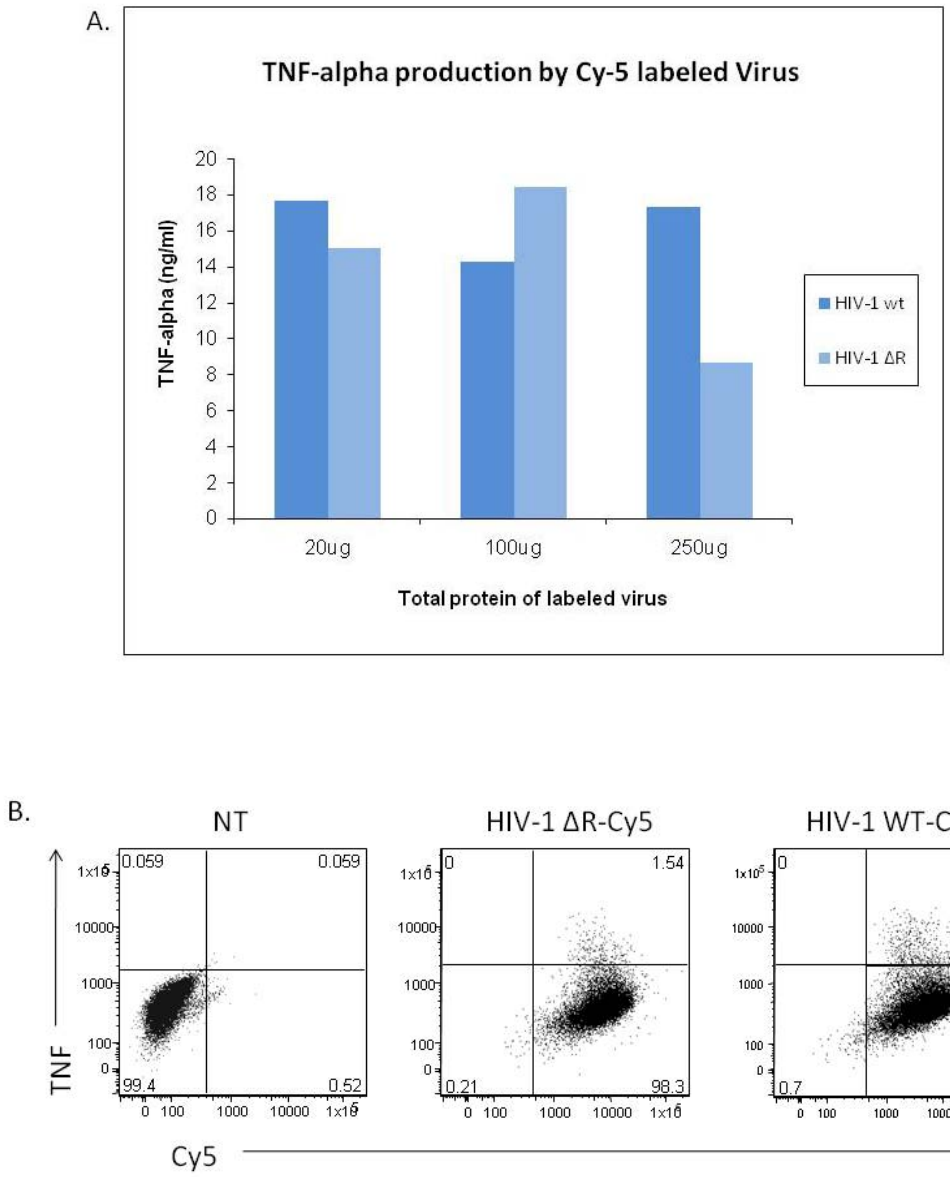



**Figure 14:** ***Detection of Soluble TNF-α in DC supernatants.***
**Cells were cultured as described and exposed to Cy-5 labeled virus for 48 hours and stimulated with LPS for an additional 24 hours. (A) Supernatants were collected and the presence of soluble TNF-α was detected by ELISA. (B) Cells were stimulated with LPS for six hours with GolgiStop and stained for TNF-α and analyzed by FACS analysis (representative of three donors).**

To control for the increase in TNF-α seen by exposure to Cy5 labeled HIV-1 WT, IL-15 was detected by flow cytometry as well. The expected trend of decreased surface IL-15 in the presence of labeled HIV-1 Vpr(+) virus compared to Vpr-defective virus was seen, representing that TNF-α production by Vpr is a specific effect (Figure 15). This also shows evidence of virus functionality after the labeling process.

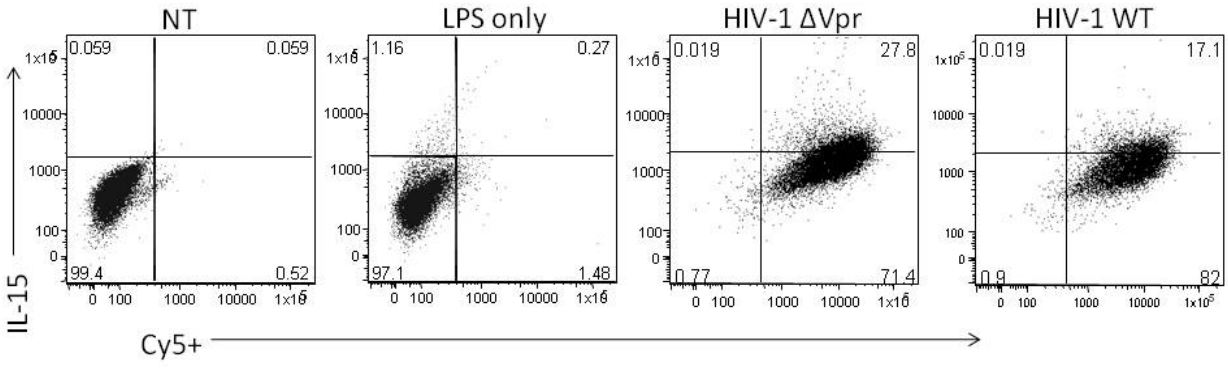



**Figure 15:** ***IL-15 modulation by HIV-1 Vpr (+) and Vpr (-) Cy-5 labeled virus.***
**DCs were cultured as described and exposed to Cy-5 labeled HIV-1 WT or HIV-1 ΔVpr virus (250µg total protein/ml) for 48 hours and additionally stimulated with LPS for 24 hours. Cells were harvested and stained for surface IL-15 expression which was quantified by FACS analysis.**

## 5.2 AIM #2: TO DETERMINE THE MECHANISMS IN THE REGULATION OF TNF-ALPHA EXPRESSION BY HIV-1 VPR

### 5.2.1 Introduction

In our laboratory and in others, it has been well established that HIV-1 Vpr transactivates several viral and cellular genes [12, 19]. We also know that Vpr is involved in the upregulation of TNF-α as virion associated and as free Vpr at the transcriptional level [19]. To further understand the transcriptional regulation of TNF-α by Vpr, transactivation assays were performed using full-length and deletion mutant TNF-α promoter constructs placed upstream of the firefly luciferase reporter gene. Upon activation of the TNF-alpha promoter, production of the luciferase enzymatic protein occurs and can be detected using a luciferase substrate kit. Transactivation was measured by relative light units (RLU).

### 5.2.2 Assay Parameters

To investigate Vpr and the transactivation of the TNF-alpha promoter, we used a full length and deletion mutant promoter sequences constructed directly upstream of firefly luciferase gene as shown (Figure 16). The full length promoter construct is -958bp and includes transcription factors SP1 and NFκB which have been shown previously to interact with Vpr.

A.

TNF-Promoter Constructs

>958

GCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGATGGCCGACATGTAGCGGCTCTGAGGAATGGGTTACAGGAGACCTCTGGG
GAGATGTGACCACAGCAATGGGTAGGAGAATGTCCAGGGCTATGGAAGTCGAGTATGGGGACCCCCCCTTAACGAAGACAGGGCCATGTAGAGGGCCCCAG
GGAGTGAAAGAGCCTCCAGGACCTCCAGGTATGGAATACAGGGGACGTTTAAGAAGATATGGCCACACACTGGGGCCCTGAGAAGTGAGAGCTTCATGAAA
AAAATCAGGGACCCCAGAGTTCCTTGGAAGCCAAGACTGAAACCAGCATTATGAGTCTCCGGGTCAGAATGAAAGAAGAAGGCCTGCCCCAGTGGGGTCTG
TGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCTGCTACCCCCACCCAGCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCC
CAGCTCCTTCTCCCCGCAGGGACCCAAACACAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAAGGACTCAGCTTTCTGA
AGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAG
GTTTTGAGGGGCATGGGGACGGGGTTCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGCAGGGAGGATGG
GGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCGCCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTA
CCGCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAA

>732

……..CTAGTTGGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCTGAGAAGTGAGAGCTTCATGAAAA
AAAATCAGGGACCCCAGAGTTCCTTGGAAGCCAAGACTGAAACCAGCATTATGAGTCTCCGGGTCAGAATGAAAGAAGAAGGCCTGCCCCAGTGGGGTCTG
TGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCTGCTACCCCCACCCAGCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCC
CAGCTCCTTCTCCCCGCAGGGACCCAAACACAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAAGGACTCAGCTTTCTGA
AGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAG
GTTTTGAGGGGCATGGGGACGGGGTTCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGCAGGGAGGATGG
GGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCGCCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTA
CCGCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAA

>605bp

AGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCTGCTACCCCCACCCAGCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCC
CAGCTCCTTCTCCCCGCAGGGACCCAAACACAGGCCTCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAAGGACTCAGCTTTCTGA
AGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAGTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAG
GTTTTGAGGGGCATGGGGACGGGGTTCAGCCTCCAGGGTCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTCGGAATCGGAGCAGGGAGGATGG
GGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCGCCCCCGCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTA
CCGCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAA

>91bp

TTACAAATAGCTCAGATCCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAA

>25bp

TTTTTTTCACTGCATTCTAGTTGGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCAAGCTCAGATCCCTCTCGCCCCAGGGACATATAAA

B.

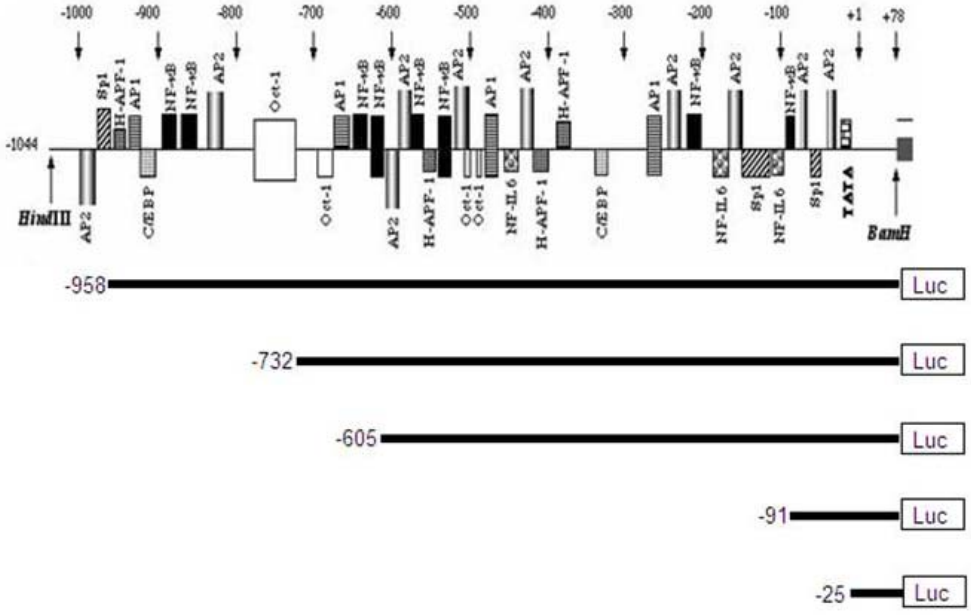



**Figure 16:** ***Representation of TNF-alpha Promoter and Deletion Mutants.***
**(A) Sequences highlighted in green indicate those that are homologous to the full-length promoter (-958) ending at the TATA box. (B) A schematic depicting the promoter constructs and their transcription factor orientations.**

Initial experiments were performed in order to determine the optimal DNA concentration of Vpr plasmid for transfection, which will result in optimal transactivation of the TNF-alpha promoter without inducing apoptosis. The concentration of the full length promoter-luciferase construct (-958) remained constant (1μg) and the concentrations of the Vpr expression plasmid (pVpr) or an empty vector plasmid (pVector) ranged (0.1 μg, 0.2 μg, 0.5 μg, 1.0 μg) as seen in Figure 17 and 18. For consistency, LPS was used for stimulation as transactivation could not to be distinguished with expression plasmids alone (data not shown). PMA and ionomycin was used for stimulation in HeLa T4 cells because they do not express TLR4 and are unresponsive to LPS.

In macrophage derived microglia cells (Figure 17A), promoter activation by Vpr remained steady but greatly dropped above 0.5μg. This could be a result of cytotoxicity by Vpr at higher concentrations as a great amount of cell death was observed in culture by microscopy. To further analyze the lower DNA concentrations for optimization, transactivation by the empty expression vector was subtracted from that by pVpr. This revealed 0.5μg to be the best for use in these experiments. Similar results were observed in HeLa T4 cells, with transactivation by Vpr greatly decreasing at 1.0 μg and 0.5μg showing to be the optimal DNA concentration of pVpr or pVector for transfection (Figure 18). This selected concentration was used in all further promoter transactivation experiments.

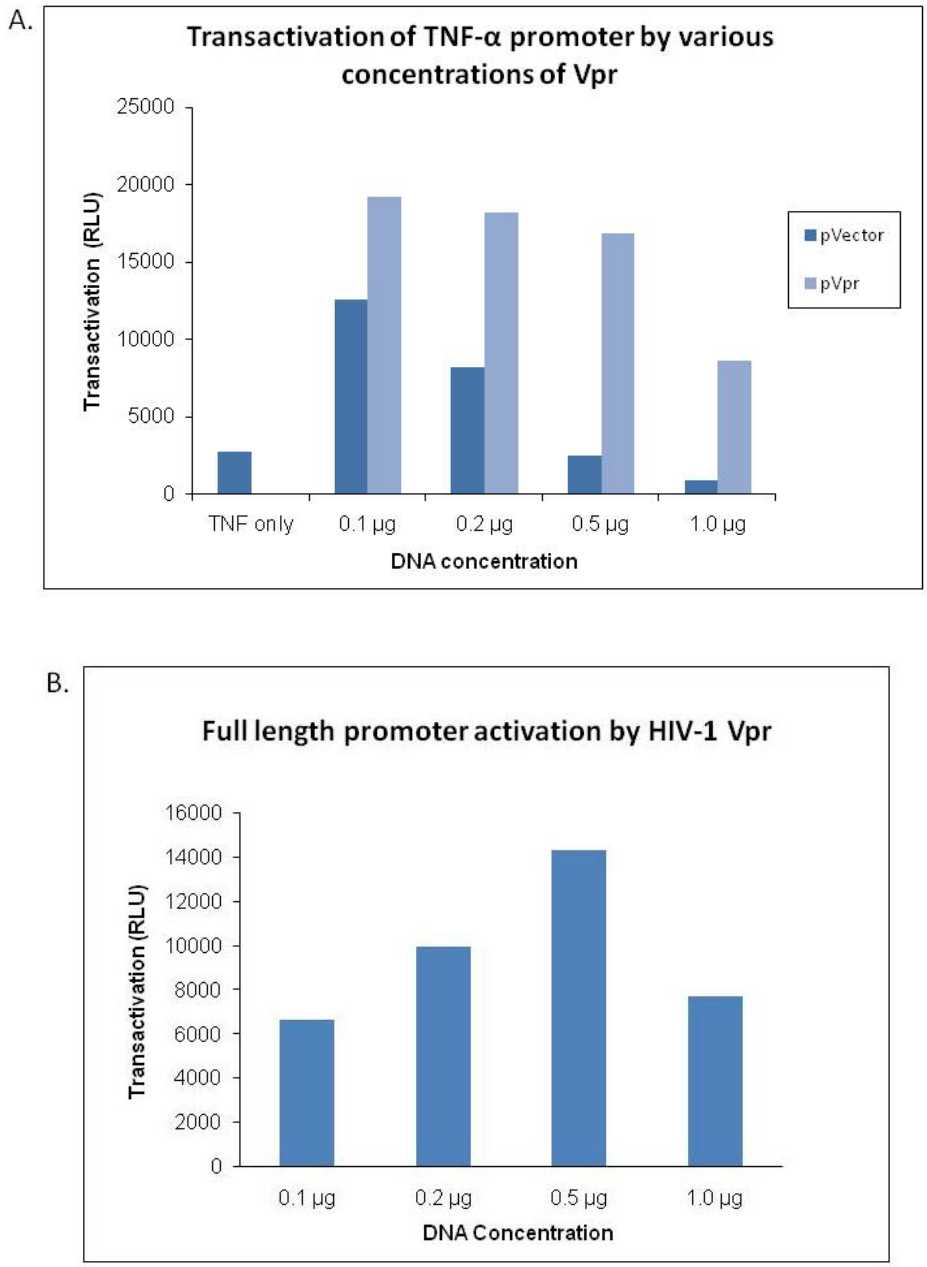
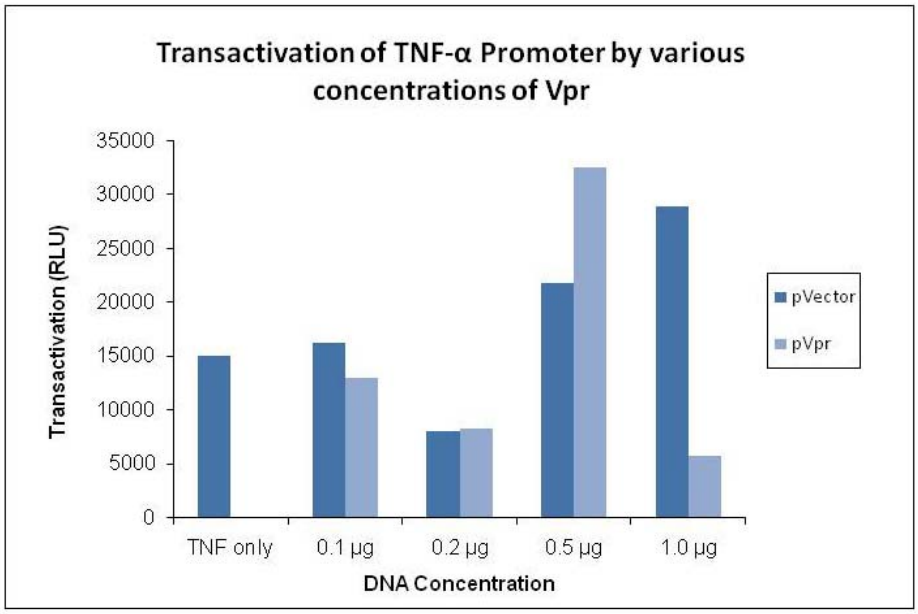



**Figure 17:** ***Assay Parameter Development using the full length TNF-α promoter in Microgila cells.***

**(A) Various concentrations of pVpr or pVector were cotransfected with the -958bp full length TNF-α promoter-luciferase construct. Forty eight hours post transfection, promoter transactivation was detected by luciferase reporter assay. (B) Transactivation by pVpr was measured by subtracting the RLU values observed in pVector transfected lysates from pVpr transfected lysates.**

A.




B.

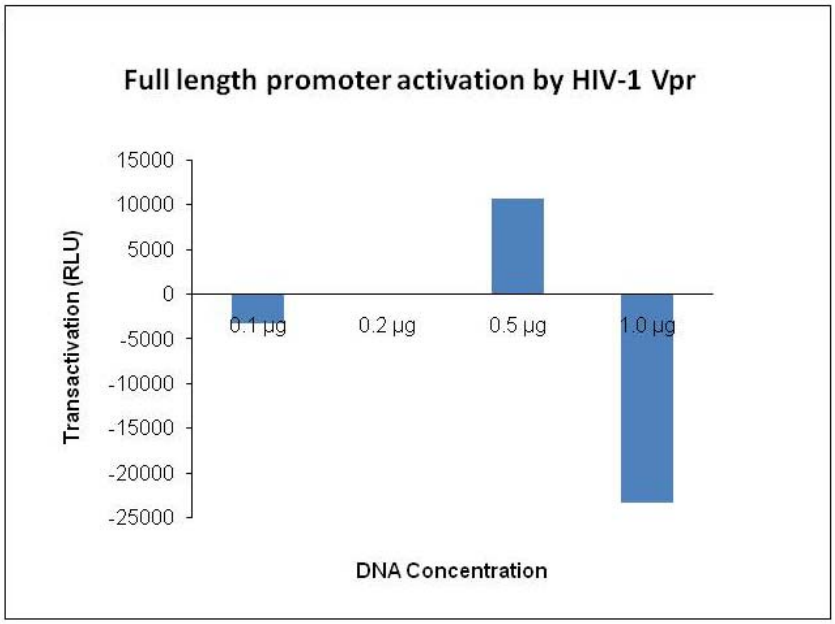



**Figure 18:** ***Transactivation of TNF-alpha by Various Concentrations of HIV-1 Vpr in HeLa T4 cells.***

**HeLaT4 cells were co-transfected with the TNF promoter and varying concentrations of pVpr or pVector. (A) Forty-eight hours post-transfection, cell lysates were collected and promoter transactivation was determined as RLU. (B) Transactivation by pVpr was measured by subtracting the RLU values observed in pVector transfected lysates from pVpr transfected lysates.**

### 5.2.3 Promoter activation by HIV-1 Vpr in Microglia cells

Due to the apparent induction of TNF-alpha in the presence of Vpr, and Vpr's ability to regulate host and viral gene expression at the transcriptional level, it was important to study the transcriptional regulation of the TNF-α promoter. Transactivation assays were performed using a full length and four additional truncated promoter constructs. As shown in Figure 19, promoter activation by Vpr occurs at the highest level when the full length promoter is present. Fold activation of the truncated promoters still occurred, though reduced by half. These results point out that in order for Vpr to fully activate TNF-α production, presence of the full length promoter is necessary.

**Table 3:** ***TNF-α Promoter Activation (RLU) in Microglia Cells.***

| Constructs Used | pVPR +LPS | pVector +LPS |
|---|---|---|
| -958 | 4307 | 690 |
| -732 | 845 | 598 |
| -605 | 3172 | 887 |
| -91 | 2543 | 947 |
| -25 | 3475 | 1262 |

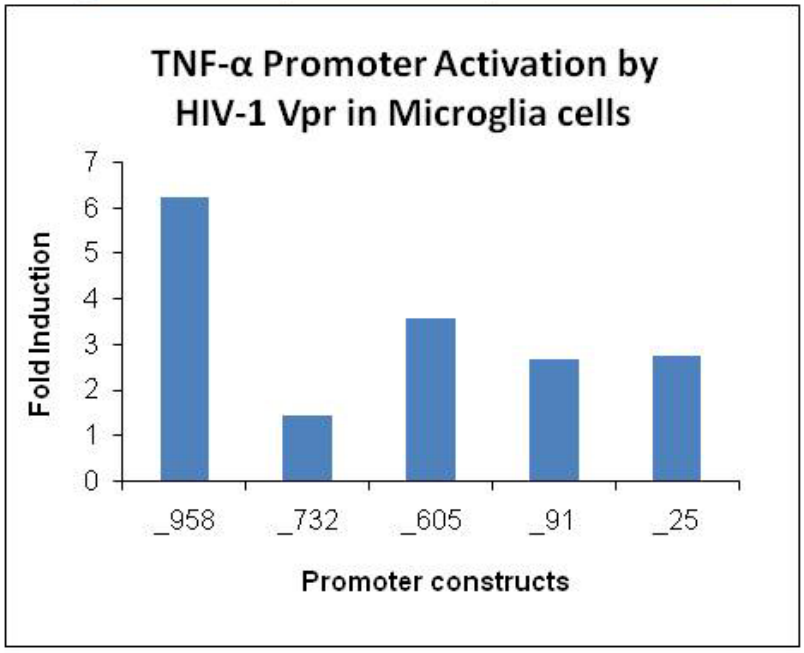



**Figure 19:** ***Fold activation of the TNF-α promoter by HIV-1 Vpr in Microglia cells.***
**Cells were co-transfected with said promoter construct and pVpr or pVector expression plasmids. Cells were stimulated 24 hours post-transfection with LPS and cell lysates were collected. This figure is representative of five independent experiments.**

### 5.2.4 Promoter induction by HIV-1 Vpr in HeLa T4 cells

To further look at the activation of the TNF-α promoter by HIV-1 Vpr, a second transactivation assay was performed using the HeLa T4 cell line and whole virus. HeLa T4 cells express CD4 and can be productively infected. This allowed for insight in the promoter's interaction with Vpr in the context of infection. Experiments were completed in the same manner; however promoter constructs were transfected alone. HIV-1 Vpr(+) or HIV Vpr(-) was added with PMA and ionomycin 24 hours post-transfection.

**Table 4:** ***TNF-α Promoter Transactivation (RLU) in HeLa T4 Cells.***

| Constructs Used | HIV-1 Vpr(+) +PMA/iono | HIV-1 Vpr(-) +PMA/iono |
|---|---|---|
| -958 | 68764 | 33718 |
| -732 | 58956 | 22059 |
| -605 | 12659 | 10239 |
| -91 | ND | ND |
| -25 | ND | ND |

Interestingly, transactivation was apparent with the full-length promoter as well as the -732bp and -615bp truncated mutants, but was not seen in the -91bp and -25bp mutants (Figure 20). Fold increase of transactivation by HIV-1 Vpr(+) compared to HIV-1 Vpr(-) is shown below. This supports previous experiments in that Vpr can induce TNF-α production by interaction with the downstream domains of the promoter however, maximum activation occurs in the presence of the full length promoter.

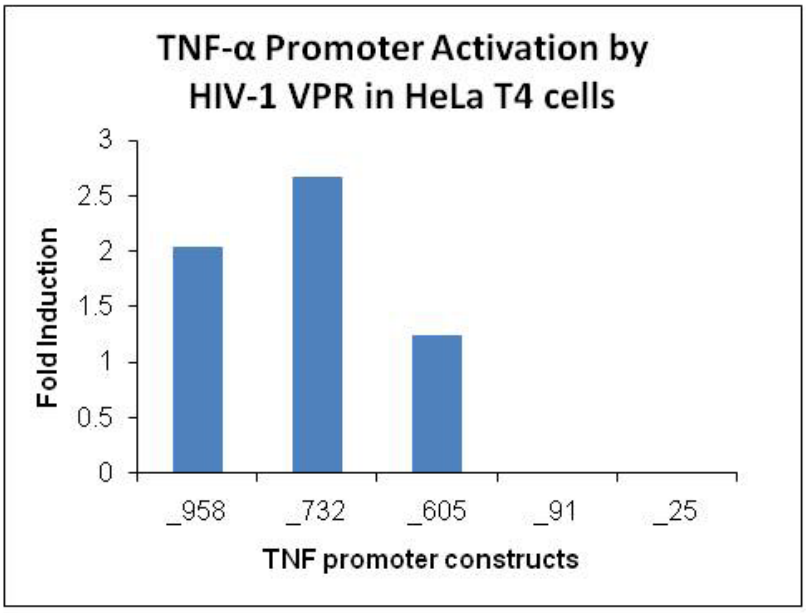



**Figure 20:** ***Fold activation of the TNF-α promoter by HIV-1 Vpr in HeLa T4 cells.***
**Cells were co-transfected with said promoter construct. Cells were stimulated 24 hours post-transfection with PMA and ionomycin in addition to NL43 HIV-1 WT or ΔVpr and cell lysates were collected. This figure is representative of three independent experiments.**

# 6.0 CHAPTER SIX: DISCUSSION

In the more than twenty-five years since the start of the HIV/AIDS epidemic, scientists have faced constant challenges in discovering therapeutics and developing an effective vaccine. One large barrier has been the ability of viral proteins to interact with host encoded components. This can occur through direct interaction or indirectly by signaling. It has been proposed that this is a possible factor for viral pathogenesis and its ability to invade immunologically privileged areas of the body including the lymph nodes and the brain. TNF-alpha has proved to be an important component in HIV-associated dementia in AIDS patients as well as other ailments like rheumatoid arthritis and advancement of opportunistic infections [13, 27-29]. Vpr has also been found in brain tissue of infected patients [30]. This provides important relevance for understanding how HIV-1 Vpr affects TNF-α and the cell types that are involved.

As seen in Figures 6, 7, 9, and 10, it is apparent that HIV-1 Vpr plays a role in the induction of TNF-α in dendritic cells. The levels of TNF-α detected by ELISA and flow cytometry are significantly increased in the presence of Vpr containing virus or recombinant Vpr protein compared to the absence of functional Vpr. However, this was not observed unless cells were additionally stimulated by LPS. This could indicate that Vpr effects TNF-α by interaction with the Toll-like Receptor 4 (TLR4) signaling cascade for which LPS acts. Alternatively, Vpr

might act as a coactivator of TNF-α transactivation, as shown previously for other cellular factors [7, 31].

To identify the domains of Vpr responsible for the induction of TNF-α, monocyte derived DCs were exposed to six peptide pools covering the 96-amino acids of the Vpr protein. In this setting, there was no significant difference between the levels of expression of soluble TNF-α in the presence of these peptide subsets in comparison to that induced by irrelevant peptide exposure (Figure 11). This indicates that Vpr has multiple domains that have a role in increased TNF-α expression in dendritic cells. It would be interesting to compare this data to TNF-α production by intact Vpr and to also look at these peptide sets structurally to see if the conformation plays a role it their ability to induce this effect.

Next, we attempted to identify the specific population of the monocyte-derived DC cultures responsible for the increase in TNF-α production. Because it has been previously shown that TNF-α production by HIV-1 exposed, particularly HIV-1 Vpr(+) virus, DCs affects bystander T-cell subsets, it is unknown if DCs are acting from infection or simply exposure to virus. If deciphered, this would also allow for further understanding of the overall immunology of dendritic cells during HIV-1 infections and the effects they may have on each other and on other cells. We were unable to conclusively determine the DC subset responsible for the enhanced TNF-α in the context of infection by intracellular staining however by using Cy-5 labeled virus, results indicate that TNF-α production is enhanced in virus exposed cells (Figure 14).

Issues with intracellular staining were addressed in multiple ways in order to produce dependable results. First, cells were stained with TNF-α and p24 (Gag) antibodies simultaneously. The p24 antibody was staining cells not exposed to virus and therefore showed nonspecific binding. This was addressed by staining DCs in a step-wise fashion however, either way, p24 was the dominant signal seen using a flow cytometer. Because the p24 and TNF-α antibodies were both anti-mouse and there is no alternate antibody for p24 available, an anti-rat antibody for TNF-α was used in replacement. This and use of alternate permeablization methods did not change the outcome. Further assays need to be developed to specifically differentiate infected or uninfected DCs and their separate cytokine profiles.

The Cy5 labeled viruses were used as an alternative way to detect infected cells by flow cytometry allowing intracellular staining for TNF-α without the competition of any other antibody. Though TNF-α producing Cy5 positive cells were able to be detected, several limitations inhibit us into drawing conclusions. One factor is that during the labeling process, the total protein detectable in the virus diminished as well as p24 levels becoming low. Therefore the amount of actual virus used was predictably less than in previous experiments. Another issue was that the DCs in culture became increasingly positive for Cy5 with increased added virus. This was expected, however, since the 250μg was used in the later studies, it did not allow us to look at the unexposed DC population. Also, the overall TNF-α induction was low in comparison to previous experiments.

Another aspect of HIV-1 Vpr and its differential regulation on TNF-α production can be addressed by determining the domains of the TNF-α promoter involved in activation. To do this,

we first analyzed TNF-α promoter activation by Vpr using a luciferase reporter assay in two ways. First, TNF-α promoter-luciferase constructs were co-transfected with a Vpr or empty expression plasmid in a monocyte derived cell line. An increase in promoter activation was observed using all promoter constructs by Vpr; however the strongest signal was seen in the presence of the full length TNF-α promoter. This is probably due to the lack of required transcription factors of the promoter or simply the dysfunction of the promoter itself due to its truncations.

To determine whether a similar effect could be observed during infection as well, another luciferase reporter assay was performed using the TNF-α promoter-luciferase construct transfected in HeLa T4 cells which are easily transfectable as well as infectable due to its expression of the HIV-1 receptor CD4. After transfection, cells were infected with HIV-1 NL43 WT or NL43 ΔVpr virus (100ng/ml). Activation of the TNF-α promoter by Vpr containing virus was up to four fold greater than that of Vpr deficient virus. Notably, transactivation was not observed when using the -91bp and -25bp promoter mutants. This reiterates that the full length TNF-α promoter is important for TNF-α production especially that induced by HIV-1 Vpr. Transcription factors Sp1 and NFκB might be involved in this transactivation. To further look at the specific interactions between the TNF-α promoter, experiments should be done using the promoter luciferase assay using various Vpr mutants as the method of activation.

# 7.0 CHAPTER SEVEN: FUTURE DIRECTIONS

It is clear that HIV-1 Vpr upregulates the expression of TNF-α in dendritic cells. However, it is still not clear whether this effect is seen primarily in the exposed or bystander DC population. In order to determine the particular DC population(s) responsible for the TNF-α production induced by HIV-1 Vpr, other methods can be used. One possible alternative could be to develop other virus labeling methods that are specific for viral proteins such as p24 Gag; a sort of backwards approach to traditional staining methods for flow cytometry. This way cells positive for the label could confidently be said as infected and not just randomly engulfing any labeled protein. Another approach that our laboratory has been working on is to make noninfectious virus-like particles (VLPs) containing Gag-EGFP, Vpr, and VSV-G by plasmid co-transfection. This method, theoretically, would be an ideal way to answer this question. However, there has been some difficulty with seeing a strong EGFP signal after DC uptake by flow cytometry under the FITC channel (data not shown). With successful troubleshooting of this method and use of other virus tracking technology, our question will be answered.

To further identify the specific domains of HIV-1 Vpr and the TNF-α promoter involved in TNF-α transactivation, we will combine the two approaches shown here. Cells can be transfected with the TNF-α promoter luciferase constructs along with various Vpr mutant molecules. This can help further identify the domains of HIV-1 Vpr based on differential TNF-α

promoter induction. To assess the TNF-α promoter more in depth, gel shift assays using specific transcription factor motifs should be done to understand the specific transcription factors necessary for transactivation.

## BIBLIOGRAPHY


1. *AIDS epidemic update 2007: Report fact sheet: key faces by region*. 2007, UNAIDS and WHO.

2. Barton-Knott, S. *Global HIV prevalence has levelled off*. 2007 [cited 2008; Available from: http://www.who.int/mediacentre/news/releases/2007/pr61/en/index.html.

3. Yanjie Yi, F.S., and Ronald G. Collman, *Preferential Use of CXCR4 by R5X4 Human Immunodeficiency Virus Type 1 Isolates for Infection of Primary Lymphocytes.* Journal of Virology, 2005. **79**(3): p. 1480-1486.

4. Yi, Y., et al., *Role of CXCR4 in Cell-Cell Fusion and Infection of Monocyte-Derived Macrophages by Primary Human Immunodeficiency Virus Type 1 (HIV-1) Strains: Two Distinct Mechanisms of HIV-1 Dual Tropism.* J. Virol., 1999. **73**(9): p. 7117-7125.

5. Fukumori, T., et al., *Regulation of cell cycle and apoptosis by human immunodeficiency virus type 1 Vpr.* Microbes and Infection, 2000. **2**(9): p. 1011-1017.

6. Heinzinger, N.K., et al., *The Vpr Protein of Human Immunodeficiency Virus Type 1 Influences Nuclear Localization of Viral Nucleic Acids in Nondividing Host Cells.* Proceedings of the National Academy of Sciences, 1994. **91**(15): p. 7311-7315.

7. Pavlakis, T.K.a.G.N., *Partner Molecules of Accessory Protein Vpr of the Human Immunodeficiency Virus Type 1.* DNA and Cell Biology, 2004. **23**(4): p. 193-205.

8. Emerman, M., *HIV-1, Vpr and the cell cycle.* Current Biology, 1996. **6**(9): p. 1096-1103.

9. Michael Bukrinsky, A.A., *Viral protein R of HIV-1.* Reviews in Medical Virology, 1999. **9**(1): p. 39-49.

10. Cohen, E.A., Subbramanian, R.A., and Gottlinger, H.G., *Role of auxiliary proteins in retroviral morphogenesis.* Current Topics in Microbiology and Immunology, 1996. **214**: p. 219-235.

11. Kino, T., and Chrousos, G.P., *AIDS-related insulin resistance and lipodystrophy syndrome.* Current Drug Targets - Immune, Endocrine & Metabolic Disorders,, 2003. **3**: p. 111-117.

12. Wang, L., Mukherjee, S., Jia, F., Narayan, O., and Zhao, and L.J., *Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and transactivation of viral long terminal repeat.* The Journal of Biological Chemistry, 1995. **270**: p. 25564-25569.

13. Herbein, G. and K.A. Khan, *Is HIV infection a TNF receptor signalling-driven disease?* Trends in Immunology, 2008. **29**(2): p. 61-67.

14. Tungaturthi, P.K., et al., *Role of HIV-1 Vpr in AIDS pathogenesis: relevance and implications of intravirion, intracellular and free Vpr.* Biomedecine & Pharmacotherapy, 2003. **57**(1): p. 20-24.

15. Baldwin, R.L., Stolowitz, M.L., Hood, L., Wisnieski, B.J., *Structural changes of tumor necrosis factor alpha associated with membrane insertion and channel formation.* PNAS, 1996. **93**(3): p. 623-631.

16. Tang, P., Hung, M-C., Klostergaard, J., *Human pro-tumor necrosis factor is a homotrimer.* Biochemistry, 1996. **35**(25): p. 8216-8225.

17. Valdez H., L.M., *Cytokines and cytokine therapies in HIV infection.* AIDS Clinical Review, 1997. **6**: p. 187-228.

18. Nancy C. Connolly, S.A.R.a.C.R.R., *Proinflammatory Cytokines in HIV disease – A Review and Rationale for New Therapeutic Approaches.* AIDS Reviews, 2005. **7**: p. 168-180.

19. Majumder, B., et al., *Dendritic Cells Infected with vpr-Positive Human immunodeficiency Virus Type 1 Induce CD8+ T-Cell Apoptosis via Upregulation of Tumor Necrosis Factor Alpha.* J. Virol., 2007. **81**(14): p. 7388-7399.

20. Wu, L. and V.N. KewalRamani, *Dendritic-cell interactions with HIV: infection and viral dissemination.* Nat Rev Immunol, 2006. **6**(11): p. 859-868.

21. Turville, S.G., et al., *HIV gp120 receptors on human dendritic cells.* Blood, 2001. **98**(8): p. 2482-2488.

22. Cameron, P.U., et al., *Dendritic Cells Exposed to Human Immunodeficiency Virus Type-1 Transmit a Vigorous Cytopathic Infection to CD4+ T Cells.* Science, 1992. **257**(5068): p. 383-387.

23. Oren J. Cohen, A.K., Anthony S. Fauci, *Host factors in the pathogenesis of HIV disease.* Immunological Reviews, 1997. **159**: p. 31-48.

24. Silvia Bulfone-Paus, E.B., Thomas Pohl, H.D.R. Vadim Budagian, Rene Ruckert, Ulrich Kunzendorf, and A.H.K. Ralf Paus, *Death deflected: IL-15 inhibits TNF-a-mediated*

*apoptosis in fibroblasts by TRAF2 recruitment to the IL-15Ra chain.* The FASEB Journal, 1999. **13**: p. 1575-1585.

25. Majumder, B., et al., *Vpr positive Human Immunodeficiency Virus (HIV-1) infection impairs NK cell function indirectly through cytokine dysregulation of infected target cells.* J. Virol., 2008: p. JVI.01979-07.

26. Maddon PJ, D.A., McDougal JS, Clapham PR, Weiss RA, Axel R., *The T4 gene encodes the AIDS virus receptor and is expressed in the immune sytstem and the brain.* Cell, 1986. **47**: p. 333-348.

27. Walker, U.A., A. Tyndall, and T. Daikeler, *Rheumatic conditions in human immunodeficiency virus infection.* Rheumatology, 2008: p. ken132.

28. Kaul M, L.S., *Mechanisms of neuronal injury and death in HIV-1 associated dementia.* Current HIV Research, 2006. **4**(3): p. 307-318.

29. Day, C., et al., *Detection of Polyfunctional Mycobacterium tuberculosis-Specific T Cells and Association with Viral Load in HIV-1-Infected Persons.* The Journal of Infectious Diseases, 2008. **197**(7): p. 990-999.

30. Wheeler, E.D.A., C.L. Achim, and V. Ayyavoo, *Immunodetection of human immunodeficiency virus type 1 (HIV-1) Vpr in brain tissue of HIV-1 encephalitic patients.* Journal of Neurovirology, 2006. **12**(3): p. 200 - 210.

31. Kino, T., et al., *Nuclear Receptor Coactivator p160 Proteins Enhance the HIV-1 Long Terminal Repeat Promoter by Bridging Promoter-bound Factors and the Tat-P-TEFb Complex.* J. Biol. Chem., 2002. **277**(4): p. 2396-2405.