DIFFERENTIAL REGULATION OF HOST CELLULAR GENE EXPRESSION BY HIV-1 VIRAL PROTEIN (VPR): IMPLICATIONS FOR HOST CELL FUNCTION

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Michelle Lynn Janket

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This dissertation was presented

by

Michelle Lynn Janket

It was defended on

December 2, 2005

and approved by

Jeremy Martinson, Ph.D.
Assistant Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Todd Reinhart, Sc.D.
Associate Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Thomas Smithgall, Ph.D.
Professor, Department of Molecular Genetics and Biochemistry
School of Medicine, University of Pittsburgh

Dissertation Advisor: Velpandi Ayyavoo, Ph.D.
Assistant Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

DIFFERENTIAL REGULATION OF HOST CELLULAR GENE EXPRESSION BY HIV-1 VIRAL PROTEIN R (Vpr): IMPLICATIONS FOR HOST CELL FUNCTION

Michelle Lynn Janket, Ph.D.
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The HIV/AIDS epidemic is one of the most important public health problems facing this generation. The failure of recent vaccine trials and growing resistance to anti-retroviral drugs underscores the need for novel therapeutic strategies. Design of such therapies will depend on a detailed understanding of the mechanism of action of the HIV-1 gene products. To further that goal, we have undertaken a detailed investigation of the HIV-1 viral protein R (Vpr). We employed cDNA microarray and antibody array analyses using isogenic virus with or without Vpr to determine the effect on host cellular gene expression. Vpr induced differential regulation of 109 cellular genes representing diverse families of signaling molecules. Two gene products, NHE1 and TNF alpha, were further studied for their potential roles in Vpr-mediated apoptosis. NHE1 expression was decreased by 50% at both the protein and mRNA levels in the presence of Vpr. Vpr-mediated NHE1 downregulation correlated with a dose dependent decrease in intracellular pH as well as a decrease in the active form of the pro-survival kinase Akt. The loss of these anti-apoptotic functions of NHE1 is proposed to contribute to the apoptotic role of Vpr. The pro-inflammatory cytokine TNF alpha may also play a part in Vpr-mediated apoptosis. Macrophages infected with *vpr*-expressing virus secreted 1.1-8.5 fold more soluble TNF alpha in response to LPS stimulation than their counterparts infected with isogenic virus lacking Vpr expression. Fold upregulation of TNF alpha directly correlated with induction of apoptosis in uninfected lymphocytes, implicating TNF alpha regulation by Vpr in bystander cell death. Two polymorphisms in the TNF alpha promoter, positions -238 and -963, were found at a higher prevalence in donors showing the lowest and highest effect of Vpr on the TNF alpha response. These results suggest that host genetic determinants may affect bystander cell death and thus the course of HIV pathogenesis. Together, the results of this study present a molecular basis for changes induced in the host cell by HIV-1 Vpr and elucidate two potential pathways for the design of anti-retroviral therapeutics targeting HIV-1 Vpr.

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AIDS	-	Acquired Immunodeficiency Syndrome
AP-1	-	Activator Protein 1
APC	-	Antigen Presenting Cell
APOBEC-3G	-	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ATCC	-	American Type Culture Collection
AVERT	-	AIDS Education & Research Trust
CA	-	Capsid
CBP	-	Creb-Binding Protein
CDC	-	Centers for Disease Control
C/EBP	-	CCAAT/Enhancer Binding Protein
CTD	-	C-Terminal Domain
DHPLC	-	Denaturing High Performance Liquid Chromatography
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribonucleic Acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
ER	-	Estrogen Receptor
FBS	-	Fetal Bovine Serum
GC	-	Glucocorticoid
GM-CSF	-	Granulocyte Macrophage Colony Stimulating Factor
GR	-	Glucocorticoid Receptor
GRE	-	Glucocorticoid Response Element
HAD	-	HIV Associated Dementia
HAT	-	Histone Acetyl Transferase
HIV	-	Human Immunodeficiency Virus

HPA	-	Health Protection Agency
HRP	-	Horse Radish Peroxidase
HSF-1	-	Heat Shock transcription Factor 1
HSP	-	Heat Shock Protein
IN	-	Integrase
INR	-	Internally Normalized Ratio
IRB	-	Institutional Review Board
IUPAC	-	International Union of Pure and Applied Chemists
JNK	-	c-Jun-Terminal Kinase
LPS	-	Lipopolysaccharide
LTA	-	Lymphotoxin A
LTR	-	Long Terminal Repeat
MA	-	Matrix
MCSF	-	Macrophage Colony Stimulating Factor
MHC	-	Major Histocompatibility Complex
NC	-	Nucleocapsid
NES	-	Nuclear Export Signal
NHE1	-	Sodium Hydrogen Exchanger, isoform 1
NIH	-	National Institutes of Health
NIH-ARP	-	NIH Aids Research and Reference Program
NLS	-	Nuclear Localization Signal
NNRTI	-	Non-Nucleoside Reverse Transcriptase Inhibitor
NPC	-	Nuclear Pore Complex
NRTI	-	Nucleoside Reverse Transcriptase Inhibitor
PARP	-	Poly(ADP-Ribose) Polymerase
PBL	-	Peripheral Blood Lymphocytes
PBMC	-	Peripheral Blood Mononuclear Cells
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PI	-	Protease Inhibitor
PIC	-	Pre-Integration Complex

Pol	-	Polymerase
PPAR	-	Peroxisome Proliferation Antagonist Receptor
PR	-	Protease (In most contexts referring to HIV in this manuscript)
PR	-	Progesterone Receptor (When referenced to Vpr LXXLL binding only)
Rev	-	Regulator of Virion protein
RNA	-	Ribonucleic Acid
RRE	-	Rev Response Element
RT	-	Reverse Transcriptase
RXR	-	Retinoic Acid Receptor
SAP	-	Shrimp Alkaline Phosphatase
SIV	-	Simian Immunodeficiency Virus
SNP	-	Single Nucleotide Polymorphism
Tat	-	Tran-Activator of Transcription
TNF	-	Tumor Necrosis Factor
Vif	-	Virus Infectivity Factor
Vpr	-	Viral protein R

PREFACE

The most important acknowledgement I have is to Dr. Ayyavoo for her support and encouragement through the years. You have not only been my dissertation advisor, but also my mentor, supporter, critic, and friend. You encouraged me to expand my scientific thinking, and with that has come an entirely new world of thinking. I know I would not be a scientist today had you not been there for me at many steps of the way. Your enthusiasm was contagious and your patience was beyond what even you imagined your patience could be. I hope that my future success will reflect on the excellent tutelage I received while in your laboratory. I am only beginning to realize now, as I move onto the next steps in my career, how much I have benefited from your teaching. Thank you!

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1.0 CHAPTER ONE: INTRODUCTION

Growing up in the 1980s and 1990s, my childhood coincided with the beginning of the AIDS crisis. My generation grew up alongside this struggle and carry memories of its earliest victims. The biggest problem to overcome at that time was a lack of knowledge about the disease. Researchers knew very little about the cause, spread or potential treatment of the disease known as Acquired Immune Deficiency Syndrome (AIDS). The discovery of a novel virus thought to be responsible for AIDS in 1983 (Barré-Sinoussi, 1983, Popovic, 1984) provided the platform for aggressive research by scientists all over the world into the workings of this pathogen. According to Dr. Robert Gallo;

> "The period after the May 1984 publication of our papers was marked by rapid advances. The HIV-1 genome was sequenced, HIV antigenic variation was discovered, the virus was found in the brain of AIDS patients, genomic sequence variation was found in viral populations from the same patient, macrophages were found to be targets for HIV, various modes of HIV transmission were elucidated, all of HIV's genes and most of its proteins were defined, and the blood supply in most developed nations was rendered safe as a result of screening for HIV. Next, came identification of the HIV receptor (CD4), the discovery of SIV in chimps, and the development of the first anti-HIV drug, AZT" (Gallo, 2002).

This early frenzy of research fueled optimism that the scientific community would "have a vaccine [against AIDS] ready for testing in about two years" (Heckler, 1984). Despite this early promise, research still continues 20 years later. No vaccine trial has yet provided full protection. This has prompted the scientific community to return to studying the manner in which the virus manipulates the host in order to design more effective therapeutic and vaccine strategies. In order to fight the virus, we must first understand it. Thus, this study presents work on the effects of one of the HIV proteins, Viral Protein R (Vpr), on the host cell. The results of this study will be expected to add to the information on host cell manipulation by HIV-1, thus providing further weapons in the fight against this disease. Perhaps those of us who grew up alongside AIDS will also see its eradication in our lifetimes.

The introduction that follows is presented in order to place the current study in the context of the AIDS crisis and existing research, such that we may better understand its implications. This section will address several key questions, including;

- What is the extent of the HIV/AIDS epidemic?
- What is the genome organization of HIV and how does each gene contribute to viral replication and disease progression?
- What is the role of the HIV-1 protein studied here, Vpr, in HIV replication and disease progression?

1.1 AIDS AS A GLOBAL HEALTH PROBLEM: STATISTICS

Despite the efforts of public health groups and scientific advances over the past 20 years, the statistics on AIDS and HIV infection continue to grow. In 2004, there were an estimated 4.9 million new infections worldwide. This brought the estimated prevalence of the disease to 39.4 million people. An even more solemn statistic is the mortality caused by this disease, which reached 54 million in 2004, with 3.1 million deaths that year (AVERT.org, 2005). While worldwide prevalence is estimated to be 1.1%, this figure masks vast disparities between regions of the world. Sub-Saharan Africa continues to bear the greatest burden of disease, with an overall prevalence rate of 7.4% of all adults infected, amounting to almost 2/3 of the world's AIDS cases. Within this region, several countries are dealing with a severe epidemic, including South Africa and Zimbabwe, both with 30% of their population infected (UNAIDS, 2004).

Sub-Saharan Africa is not the only region suffering a growing epidemic. According to the UNAIDS 2004 report (UNAIDS), the number of people living with AIDS has risen in all regions of the world. The steepest increases have been in Central America, Eastern Europe, and East Asia. The growing epidemic in China accounts for much of the increase in East Asia, while that in the Russian Federation and former Russian states such as Ukraine and Estonia accounts for the increase in Eastern Europe. Yet, these statistics should not mislead us, this is not just a problem of developing and transitional countries. In 2003 there were 43,171 newly diagnosed AIDS cases in the United States. At the end of 2003 it was estimated that 1 million people were living with AIDS in this country, yielding a 0.4% prevalence rate (CDC, 2004). In the United

Kingdom, there were 6,606 newly diagnosed HIV patients in 2003 (HPA) and 7,275 in 2004 (AVERT.org, 2005), a clear upward trend. The overall prevalence rate in Western and Central Europe is 0.3% (UNAIDS). Thus even those nations at the forefront of development are affected by the AIDS epidemic, indicating that AIDS is not a problem of any one region or country, it has become a public health problem that the world must face.

The AIDS epidemic is not growing for lack of response from the nations of the world. Global funding has increased from roughly US$ 2.1 billion to an estimated US$ 6.1 billion in 2004 (UNAIDS, 2004). This figure is still a gross underestimate of what is truly spent on fighting AIDS, since it takes into account only certain categories of money. The United States' fiscal year budget for 2006, for example, includes $21 billion in spending for domestic and international AIDS. This is an increase over the 19.7 billion allotted for 2005 (Kaiser Family Foundation, 2005). The world has responded to the AIDS epidemic. The response has included education programs, research, and public health practice changes. In total, this money is spent not only on care of AIDS patients, but in significant amounts on research and prevention. The question remains then: how, despite our best efforts, does this disease continue to infect at an astonishing rate? Some of these answers may lie in the molecular biology of the virus itself.

1.2 HIV GENOME ORGANIZATION*

Human Immunodeficiency virus Type 1, the causative agent of AIDS, belongs to a Family of viruses known as *Retroviridae* or Retroviruses. Retroviruses are enveloped RNA viruses. Distinguishing features of this family of viruses are a life cycle which includes both reverse transcription of the retroviral RNA genome into DNA, which is then integrated into the host genome. Reverse transcription, which is found rarely in eukaryotic cell biology, has proven an important target of anti-retroviral therapy. Within the family *Retrovirdae*, HIV belongs to the genus *Lentiviridae*, or Lentiviruses. This genus includes several viruses, such as BIV (Bovine

* Unless otherwise noted, information in this section is from Coffin, *Retroviruses*, Fields, *Virology*, and Frankel and Young, 1998.

Immunodeficiency Virus) and FIV (Feline Immunodeficiency Virus), which cause similar immune deficiencies in other animals, as well as a number of other viruses. Simian Immunodeficiency Virus (SIV) is the non-human primate lentivirus most closely related to HIV-1. Many theories of the origin of HIV show it having evolved from SIV. Thus, SIV has proved a useful experimental model of HIV pathogenesis. HIV-1 and HIV-2 are the only known human lentiviruses, with HIV-2 causing a less pathogenic syndrome than HIV-1.

HIV is compromised of by nine genes, with a total genome size of approximately 9.9 kilobases. This is miniscule compared to another human scourge, smallpox, which is encoded for by a virus with a 190 kilobase genome. Considering the global impact of AIDS and the devastating effects of the virus on host immune function, this tiny foe packs an immense punch. HIV replication is highly error prone due to the reduced fidelity of the reverse transcription step (Bebenek 1989). Thus, HIV undergoes rapid evolutionary selection, which would be expected to select for the genomic variants having the greatest impact on replication. HIV's nine genes remain evolutionarily conserved, indicating an important role for each gene in virus replication and pathogenesis. Three of the genes, *gag, pol*, and *env*, are the structural genes shared with all Retroviruses. The regulatory genes, *rev* and *tat*, are shared among Lentiviruses. The remainder of the genes are dubbed accessory genes; *nef, vif, vpu*, and *vpr*. The following sections will give a brief overview of the role of each of these genes in the life cycle and pathogenesis of HIV-1. A more detailed treatment of the *vpr* gene product will be given in order to provide a background for the remainder of this study.

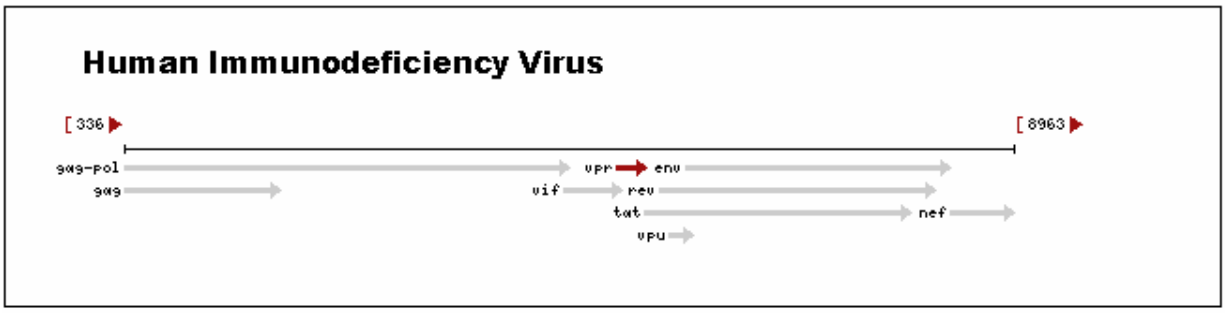

Figure 1: HIV Genome.

The nine genes encoding for the proteins of HIV-1 are shown above. Numbers denote base pair within the HIV-1 genome, shown devoid of the flanking LTR regions. Emphasis is placed on the vpr gene, which will be the subject of this study. Figure taken from, NCBI public server, Entrez Gene, Accession #155807

1.2.1 Gag

Gag, group associated antigen, encodes for the structural components necessary for assembly of HIV virions. Gag is synthesized as a polypeptide, referred to by its molecular weight p55, from an unspliced RNA product transported from the nucleus via Rev. Proteolytic cleavage of the p55 precursor by the virally encoded protease yields these three proteins, referred to most often by their molecular weights; p24- Capsid (CA), p17- Matrix (MA), and p19- Nucleocapsid (NC). The structure of the mature HIV virion can be likened to a set of nesting dolls, with several layers beneath the outer envelope. Matrix, capsid, and nucleocapsid compose each of these layers, respectively, from the outside in.

The p17 Matrix is located just below the viral envelope, forming the second layer of the virus. Beyond its structural role, MA a member of the pre-integration complex (PIC) which facilitates entry of the viral genome into the nucleus. It remains controversial whether MA possesses a nuclear localization signal, as a recent study attributed the karyophilic properties of the PIC to Integrase (IN) and Vpr (Depienne, 2000). MA is also important in targeting Gag and Gag-Pol to the plasma membrane in virus assembly (Yu, 2002).

The Capsid protein (p24) is the next nested layer, forming an inner core within the virion. Capsid is the most abundant viral protein synthesized and thus functions as an easily detectable marker for virus infection and replication. In this study, virus infection is assessed by flow cytometry using an anti-p24 antibody and virus replication is measured via p24 antigen capture ELISA. CA is also important in complexing with the cellular protein Cyclophilin A (Endrich, 1999), which is carried in the virion and has been shown to be important in modulation of host cell permissiveness to HIV-1 infection (Towers, 2003, Berthoux, 2005).

The Nucleocapsid (NC) protein comprises the remaining inner layer of the virus, forming a protective coat around the RNA genome. NC binds, recruits, and maintains the RNA genome in the virion. NC contains two zinc finger motifs, similar to other nucleic acid binding proteins, which allow it to associate nonspecifically with many regions of the HIV-1 genome (Dannull 1994, Buckman 2003). Mutations in these zinc finger regions induce packaging of foreign RNA, indicating that the specificity of HIV genome incorporation is conveyed through the NC zinc finger motifs (Mark-Danieli, 2005).

The remaining portion of the p55 polypeptide is the p6 protein. This protein is thought to play a role in virion release (Demirov 2002). In addition, and of relevance to our work, p6 is the binding partner for Vpr, mediating its incorporation into the virion (Paxton 1993, Kondo, 1995). We will touch more upon the properties of Vpr as a virion associated protein in following sections.

1.2.2 Pol

Pol, polymerase, is synthesized as a p160 polyprotein which encodes for three important enzymes: Reverse Transcriptase (RT), Integrase (IN), and Protease (PR). RT is the reverse transcriptase of HIV. This is the gene product that conveys to HIV and other retroviruses their unique ability to reverse transcribe RNA to cDNA. RT is necessary to reverse transcribe the RNA genome carried in the HIV virion into cDNA for integration into the host DNA. It is carried in the genome, allowing reverse transcription of the RNA genome prior to transport into the cellular nucleus. RT is a multi-faceted enzyme, capable of both RNA and DNA dependent polymerization, as well as possessing an RNase H domain (Starnes, 1989, Schatz, 1990). Due to the fact that reverse transcription is not common in eukaryotes, RT is a target of antiviral therapeutics. Two classes of drugs target RT, non-nucleoside reverse transcription inhibitors (NNRTI) and nucleoside reverse transcriptase inhibitors (NRTI). NRTI's mimic natural nucleotides but lack the 3' hydroxyl necessary for elongation, thus acting as chain terminators, an example of which is the well-known compound AZT (Elwell, 1987). NNRTI's bind to areas outside of the active polymerization site of RT and are thought to inhibit polymerization through an allosteric mechanism (Kohlstaedt, 1992, Tantillo, 1994). Resistance to both NRTI's and NNRTI's is becoming a growing problem (mechanisms of resistance reviewed in Soriano, 2002, Vivet-Boudou, 2006).

Integrase (IN) is the enzyme responsible for catalyzing incorporation of the viral cDNA into the host genome. Following reverse transcription, described above, the viral genome, now in DNA form, is transported into the host cell nucleus as part of the pre-integration complex (PIC). Integrase is a major component of the pre-integration complex, as are several other viral proteins, including Matrix and Vpr. (HIV nuclear import reviewed in Bukrinsky, 2004). Once

inside the nucleus, integration occurs through a two-step process, with both steps catalyzed by Integrase (Mazumder, 1994). The first step, referred to as 3' processing, removes two bases from the 3' ends of the HIV genome, yielding overhanging ends. In the second step, strand transfer, the 3' hydroxyl groups of the HIV genome "attack" the phosphodiester bonds of the host DNA. The cellular DNA repair system then closes the remaining gaps of the intermediate and the HIV genome is affixed into the host chromosome (Willetts, 1999, Brin, 2000, Mulder, 2002, Faust, 2002). Replication of all retroviruses, including HIV, requires efficient completion of these integration steps (Goff, 1992). As such, integration would be expected to be an excellent target for antiviral therapeutics. Though studies have been underway for more than ten years (Mathe, 1999) and are currently receiving a renewed interest (Chi 2005, Vallano 2005), no drugs of this class are yet available. An obstacle to the design of integrase inhibitors is the lack of a complete crystal structure of HIV integrase due to its insolubility. The recent elucidation of the multidomain IN crystal structures may advance design of integrase inhibitors towards potential therapies (Chen, 2000, Wang, 2001).

The final enzyme encoded for by the *pol* gene is Protease (PR). HIV has a unique life cycle in that the virus particles budded from the cell surface during virus replication are noninfectious. These immature virions undergo processing after and potentially concurrent with budding that completes the process of formation of mature infectious HIV (Gottlinger, 1989). Protease enzyme plays a vital role in virus maturation through cleavage of the Gag and Gag-Pol polyproteins carried in the immature virion into MA, CA, NC, p6, IN, RT, and PR (Henderson, 1990). HIV particles then undergo conformational changes, resulting in the nested virus structure described above (See Section 1.2.1: Gag). Interestingly, the Gag-Pol precursor alone is sufficient for cleavage, without additional PR. This indicates that embedded PR catalyzes its own cleavage from the Gag-Pol precursor. This cleavage is initiated when two Gag-Pol polyproteins form a dimer and a recent study showed that cleavage is intra- versus inter-molecular (Pettit, 2004, 2005). The vital role of PR in producing infectious HIV has made it a target of antiviral therapy as well. While protease inhibitors (PI's) have proven effective as a class of anti-retroviral drugs, their use has also been associated with side effects such as lipodystrophy (Carr, 1998, Viraben, 1998). HIV associated lipodystrophy is a syndrome involving peripheral lipoatrophy and deposition of fat on the trunk of the body which is thought to occur as a side effect of the combined metabolic changes induced by HIV and the effects of

protease inhibitor use, most notably first generation PI's (Safrin, 1999). This syndrome, which we will touch upon again in Chapter 4, is considered to be a factor in noncompliance with anti-retroviral therapy in AIDS patients (Sweet, 2005). Thus, discerning the mechanism of the metabolic changes induced by HIV and PI's has become a major research focus in order to provide a basis for the design novel protease inhibitors without this side effect.

1.2.3 Env

Envelope (Env), like both *gag* and *pol*, encodes for a polypeptide encompassing more then one protein. Env is synthesized as the gp160 precursor protein, which is cleaved within the host cell to form gp120 (SU) and gp41 (TM). Unlike Gag and Pol, Env is cleaved by a host cellular protease, furin (Hallenberger, 1992). Following cleavage, SU and TM form a noncovalent multimeric structure. The *env* encoded proteins together are important for viral fusion to and entry into the host cell. Surface antigen, SU, initiates host cell entry by binding to CD4 at the cell surface (Lasky, 1987). In models of virus entry, SU interaction with CD4 induces a conformational change in SU, exposing its V3 loop to additional coreceptors on the cell surface. Binding of SU to both CD4 and a coreceptor, CCR5, CXCR4, and potentially others, then initiates viral envelope fusion with the host cell membrane (Xiao, 1998, Verrier, 1999). Envelope fusion is then mediated by TM through a mechanism that is not yet well understood. Although separated by proteolytic cleavage after translation, SU and TM remain a cooperative protein complex. Together, SU and TM form a trimeric complex on the surface of a HIV particle. SU is the exposed surface antigen and TM is the transmembrane region tethering SU to the particle. A new class of anti-retroviral drugs, fusion inhibitors, has arisen in part to target the action of these two proteins. Although, *env* remains the most highly variable gene of HIV, making it a moving target difficult for a therapy to hit.

1.2.4 Rev

Rev is one of the two regulatory genes carried by all Lentiviruses. As stated above, some of the HIV proteins are synthesized as polyproteins, followed by cleavage later in the life cycle. In order to synthesize genes in this manner, unspliced RNAs must exit the nucleus for translation in the cytoplasm. Normally, in eukaryotic cells, splicing must occur before an RNA can exit the nucleus. Thus, Rev binds to a special sequence in the HIV transcript, termed a Rev Response Element (RRE), and facilitates exit of the unspliced RNA from the nucleus of the cell. In order to carry out this function, Rev must shuttle between the nucleus and cytoplasm, which it does via a Nuclear Export Signal (NES) (Wen 1995, Fritz, 1996). Rev itself is produced as an early transcript, thus it is present when synthesis of the polyproteins begins.

1.2.5 Tat

Transcription of viral genes is controlled by the Long Terminal Repeat (LTR). Tat encodes for the Trans-Activator of Transcription, aptly named for its role in enhancing the transcription of viral genes from the LTR. Tat acts as a processivity factor for viral transcripts, facilitating transcript elongation (Kato 1992, Cullen 1990). In the absence of Tat, viral transcripts are short, no more than a couple hundred base pairs, due to inefficient transcription from the LTR. In the presence of Tat, polymerase becomes sufficiently processive to transcribe the entire >9kb HIV genome. Tat recognizes an RNA motif known as TAR (trans-activating response element) in the 5' end of the viral transcript (Feng, 1988, Hauber, 1988). Tat and TAR form a stable complex, and Tat is thought to recruit additional cellular transcription factors, including the Cyclin T1/CDK9 complex (Cujec, 1997, Zhoa, 2000). Cyclin T1 and cDK9 enhance elongation by phosphorylation of the CTD (C-terminal domain) of RNA Polymerase II (Isel, 1999). Studies have indicated that such phosphorylation may be sufficient to convey to RNA Polymerase the processivity associated with Tat binding (Parada, 1996, Kim, 2002). In addition to its role as in transcriptional control, Tat is shed from infected cells as a soluble protein. Cell free Tat has been postulated to function as a neurotoxin in inducing HIV Associated Dementia (HAD) (Maragos, 2003, Pu 2003).

1.2.6 Nef

Negative regulatory factor, Nef, is an accessory gene product which has been extensively studied due to its extensive role in HIV replication. Nef is a 27kD protein which has been shown to be carried in the HIV virion (Pandori, 1996, Welker, 1996). Nef has been shown to undergo N-myristolation, allowing it to localize to the plasma membrane, which has been shown to play a role in its virion incorporation (Bukovsky, 1997). Upon incorporation, Nef appears to be cleaved by HIV protease into two products, an 18-20kD product and a small N terminal domain peptide. This cleavage has thus far not been correlated with the functions of Nef (Miller, 1997, Chen, 1998).

Initial studies of Nef indicated that it may play a role in suppressing virus replication and inducing a state of viral latency, hence the name negative regulatory factor (Ahmed, 1988, Niederman, 1989). Multiple studies soon followed disputing this negative effect and showing the exact opposite effect, that Nef played an important role in increasing HIV infectivity and replication (Hammes, 1989, Kim, 1989, Chowers, 1994, Miller, 1994). Sequencing of a cohort of 10-14 year nonprogressing patients infected from the same blood donor revealed partial *nef* deletion as the only viral defect, supporting a vital role for *nef* in HIV infection (Hanna, 1998, Birch, 2001). Despite years of research, we still do nut understand the exact mechanism by which Nef increases viral infectivity (reviewed in Joseph, 2005, Marsh, 1999). It does appear that this is an early affect though, prior to or at the level of proviral DNA formation (Chowers, 1995, Schwartz, 1995).

A better understood effect of Nef is the role it plays in modulation of the immune system of the host during HIV infection. Early in its study, Nef was shown to induce downregulation of CD4 on infected cells (Yuille, 1989, Garcia, 1991). CD4 is an important component of T cell signaling in the immune response, as well as the main receptor for HIV cellular entry. Downregulation of CD4 may therefore allow the HIV infection to proceed in a cell without potentially deleterious superinfection occurring (Benson, 1993). Nef has also been shown to mediate downregulation of MHCI (Schwartz, 1996, Blagoveshchenskaya, 2002). Major histocompatibility complex class I (MHCI) is the cell surface molecule responsible for presentation of intracellular pathogen epitopes for generation of an immune response. Loss of the ability of the infected cell to display foreign antigens (MHCI) or stimulate an APC response

(CD4) may aid the virus by allowing infected cells to avoid immune surveillance and thus destruction by the immune system (Piguet, 1999, Tolstrup 2004). In addition to these two major immune molecules, Nef has also now been shown to regulate multiple immune molecules, including; MHCII (Schindler, 2003), CXCR4 (Hrecka, 2005), CD28 (Bell, 2001, Swigut, 2001), CD1a (Chen, 2005), CD80 and CD86 (Chaudhry, 2005), and the T cell receptor (Schaefer, 2002, Stove, 2005), as well as potentially others (Choi, 2003). Such severe regulation of the immune system would be expected to compromise the immune system of the host. In fact, Nef administered in the absence of HIV infection or other viral proteins causes an AIDS-like immunodeficiency in mice and primates (Hanna, 1998, Simard, 2002). These results indicate an important role for Nef in host immune modulation. Unfortunately, the effects of Nef on the host immune response have made it difficult to include in most vaccine constructs. Elucidation of the mechanisms of Nef modulation of the immune system may be one of the most important areas in current HIV research. Such information would allow for the design of therapeutics to decrease the immunocompromised state of AIDS patient. Since AIDS patients suffer and die from opportunistic infections, such treatments would be expected to greatly add to the quality of life and life expectancy of these patients.

1.2.7 Vif

Virus infectivity factor, Vif, is an accessory gene product shown to be incorporated into virions at low levels (Liu, 1995, Karczewksi, 1996). Until recently, the function of Vif in HIV replication and pathogenesis remained largely unknown. Recently, Vif has been found to be important in influencing cell permissivity for HIV infection and replication (Borman, 1995, Simon[2], 1998). Specifically, evidence now shows that Vif counteracts the anti-viral effects of the host protein APOBEC3G (Madani, 1998, Simon, 1998[1,], Sheehy, 2002). Vif's anti-viral activity appears to be through the stimulated degradation of APOBEC3G (Sheehy, 2003, Stopak, 2003, Conticello, 2003, Mehle, 2004). In addition, another similar anti-retroviral factor, APOBEC3F, has also been found to be inhibited by Vif (Weigand, 2004). These recent discoveries have prompted a deluge of new investigations into the mechanisms of APOBEC-

mediated anti-retroviral functions. Inhibition of Vif has now become an attractive target for anti-retroviral therapy (Barnor, 2005, Stopak, 2005). The next few years should bring interesting results regarding this small protein.

1.2.8 Vpu

Viral protein U, Vpu is also considered an "accessory" gene, but has been found to play two important roles in HIV replication and infection. Upon replication of viruses lacking *vpu*, the nascent virions remain in the cytoplasm and do not bud (Klimkait, 1990). Vpu was thus found to have a role in virion budding and release from the host cell membrane. At present, the mechanism through which Vpr modulates this effect remains largely undescribed. The second role for Vpu has been in CD4 degradation (Willey, 1992, Bour, 1995). SU and TM, through their inherent affinity for CD4, have been found to associate with CD4 in the endoplasmic reticulum, thus trapping them in the ER of host cells. Vpr has been found to associate with and degrade CD4, freeing SU and TM to move to the cell surface (Lama, 1999, Levesque, 2003). The mechanism of action is thought to occur through targeting to the cellular proteasome (Fujiti, 1997) and is mediated by phosphorylation of Vpu itself (Paul, 1997).

1.2.9 Vpr

Viral protein r, Vpr, is a small 96 amino acid, 14kD protein. Though classified as an "accessory" gene product, Vpr is necessary for HIV replication and pathogenesis *in vivo*. Vpr is conserved between HIV-1, HIV-2, and SIV, indicating an important role in viral replication. In HIV-2 and SIV, the functions of Vpr are divided between the *vpr* and *vpx* genes. In addition, Vpr is packaged and carried in the virion through specific interaction with p6 Gag, implicating this gene product in the immediate early steps of HIV infection (Paxton, 1993, Kondo, 1995). Several functions have been described for Vpr including; induction of cell cycle arrest in infected cells, nuclear import of the pre-integration complex, induction of apoptosis in infected and bystander cells, and transactivation of the LTR. As Vpr is the focus of the study contained herein, the remainder of this introduction will focus on each of the functions of Vpr and what is

known about the mechanism of each and their contribution to HIV replication and disease progression.

Structure of Vpr

Before describing the functions of Vpr, it is important to have a clear understanding of the structure of this protein. This will provide background for the later sections in which structural aspects of Vpr are referenced in describing the structure/function relationship of this protein. Unfortunately, due to its inherent tendency to aggregate, no crystal structure of Vpr has yet been attained. The data that are available on the structure of Vpr come mainly from NMR studies (Morellet, 2003, Schuler, 1999, Wecker, 1999). Vpr is composed of three alpha helices separated by short beta-loops. The first two helices are contained in the N terminal 1-51 amino acids of the protein (Wecker, 1999). The first helix is approximately 10-11 amino acids in length and bounded by prolines. These proline residues have been found to undergo cis/trans isomerization, potentially affecting the differential functioning of Vpr (Bruns, 2003). This helix also contains a noncanonical LXXLL motif at amino acids (22-LEELL-26), one of two LXXLL motifs in Vpr.

The leucine rich LXXLL motif is a conserved motif associated with binding to steroid hormone receptors (reviewed in Leo, 2000). The steroid hormone receptors are a family of nuclear receptors which bind steroid hormones and induce transcriptional regulation, either activation or repression of genes bearing a responsive element. Examples include the glucocorticoid (GR), estrogen (ER), progesterone (PR), mineral corticoid (MR), retinoic acid (RXR), and peroxisome proliferation (PPAR) receptors. Vpr has been shown to bind to one member of the steroid hormone receptor family, the glucocorticoid receptor (Refaeli, 1995) and to induce transcriptional regulation by GR. Mutations in either the 22-LXXLL-26 or the 64-LXXLL-68 motifs in Vpr abrogated GR binding (Sherman, 2000). Studies have not yet been published on whether Vpr is able to bind to other members of the steroid hormone receptor family which also bind LXXLL motif bearing proteins. Members of this family share ligands and coactivators/corepressors with varying affinities. The results of such experiments may prove interesting given the differences in HIV disease progression in men and women, which may potentially be regulated through the ER and PR, as well as the hypothesized effects of Vpr on lipodystrophy in AIDS patients (Kino, 2003), potentially regulated through PPAR.

The third alpha helix of Vpr is an extended helix contained within the C terminal region of the protein. This final helix is an extended amphipathic alpha-helix extending from amino acid 53-78 (Schuler, 1999). The 64-LXXLL-68 motif described above is contained within this helix. In addition, the C-terminal region of Vpr has been implicated in HIV pathogenicity, cellular protein binding, and host cell cycle arrest. All of these functions of Vpr will be described further below. Studies have shown that the functions of Vpr are separable by mutation, indicating that the domains of Vpr may function independently (Mahalingham, 1997, Nishizawa, 2000).

Nuclear Import of the Pre-Integration Complex

Contrary to its classification as an "accessory gene", Vpr has since been found to play a key role in mediating infection of nondividing host cells (Popov, 1998). One of the earliest studies indicated that Vpr was dispensable for replication in T lymphocytes (Dedera, 1989). This study, though was conducted in cells lines and primary lymphocytes stimulated to proliferate. Studies soon followed showing that in nonproliferating cells, such as macrophages and resting lymphocytes, Vpr was essential for virus replication (Hattori, 1990, Levy, 1994). A mechanism for this differential effect of Vpr was soon elucidated. In order for integration of the viral genome to occur (as described above, Section 1.2.2), the viral genome and integrase must enter the host cell nucleus. In dividing cells, the nuclear membrane breaks down and reforms during mitosis, thus the viral genome would be expected to be included in the nucleus of a certain percentage of cells when the nuclear membrane reforms. In rapidly dividing cells such as the cell lines and proliferating lymphocytes tested by Dedera et. al. (1989), this would occur often enough to allow for adequate HIV replication. In nondividing cells, the genome must find a way to cross the nuclear membrane and this is where Vpr has been found to play a role.

The viral genome is transported into the nucleus as part of a complex of viral proteins referred to as the pre-integration complex (PIC) (Miller, 1997). Vpr is one of the viral proteins which compose the PIC, along with RT, IN, MA. Together, the nucleophilic MA and Vpr proteins are responsible for nuclear localization of the PIC (Heinzinger, 1994, vonSchwedler, 1994). The role of Vpr appears to be to "dock" the PIC to the nuclear pore complex through binding to karyopherin alpha/beta and nucleoporin hCG1, constituents of the nuclear pore complex (Popov, 1998, Le Rouzic, 2002). Direct binding to the nuclear pore complex (NPC) by

Vpr may facilitate PIC entry even in low-energy cellular states by bypassing the energy dependent soluble nuclear import systems (Jenkins, 1998). This strategy would benefit HIV in the low energy cellular states present in resting T lymphocytes. More recent literature has defined within Vpr a non-conventional nuclear localization signal as well as a nuclear export signal, making Vpr a nucleocytoplasmic shuttling protein (Sherman, 2001, 2003). The decreased ability of HIV to infect nondividing macrophages when the nucleophilic properties of Vpr are mutated makes this an attractive target for anti-retroviral therapeutics. The publications cited above indicate that the effects of Vpr are most pronounced in macrophages, the initial target of HIV infection. Thus, such therapeutics, if introduced, would be expected to be most efficacious in patients acutely infected with HIV, such as health care workers with needlestick injuries. As of yet, no such therapeutics have yet been described in the literature.

Transactivation of the LTR

A function which will be addressed in Chapter 2 is the ability of Vpr to transactivate the LTR through specific binding to host cellular transcription factors. The LTR (long terminal repeat) of HIV serves as its promoter for transcription of viral genes. While Tat is considered the main transactivator of transcription, as described above, Tat has not been shown to be carried in the virion (although this has been hypothesized, Apolloni, 2003). Thus, the question remained of what transactivates the HIV LTR prior to the transcription of Tat? The answer to this was found to be Vpr. Vpr has now been shown to facilitate the recruitment of several cellular transcription factors to the LTR and transactivate viral gene expression through several transcription factor binding sites present within the LTR. First, Vpr was shown to directly bind to three molecules of the cellular transcription factor Sp1 (Wang, 1995, Amini, 2004). Transactivation then occurs through a series of three closely spaced triplicate Sp1 sites in the LTR (McAllister, 2000). As described above, Vpr has also been shown in our laboratory and others to bind to the glucocorticoid receptor (Refaeli, 1995, Sherman, 2000, Ramanathan, 2002). Interaction of Vpr with the glucocorticoid receptor then recruits GR to a GRE (glucocorticoid response element) identified recently in the LTR (Vanitharani, 2001). Mutations of the LXXLL binding motifs in Vpr which interact with GR alter transcription from the LTR (Thotala, 2004). A recent paper has now indicated that Vpr may bind and utilize several more transcription factors, including activator protein 1 (AP-1), c-Jun-terminal kinase (JNK), and NF-kappa-B (Varin, 2005). The

results concerning NF-kappa B were not surprising, as Vpr has previously been shown to alter the transcriptional activity of NF-kappa B (Ayyavoo, 1997, Roux, 2000). Specifically, Ayyavoo, et. al. indicated that Vpr-mediated regulation of NF-kappaB resulted in differential regulation of cellular genes whose transcription was controlled by NF-kappaB. This paper provided pivotal evidence for the potential regulation by Vpr on cellular gene transcription, thus providing a basis for the hypothesis of the present study. Though NF-kappaB had previously been investigated, the results of Varin et. al. indicating binding to AP-1 and JNK would present novel transcription factor families through which Vpr may be exerting its cellular affects.

In addition to recruiting transcription factors, evidence now indicates that Vpr may also recruit transcriptional co-activators to the LTR. The addition of co-activators would be expected to increase the contribution of Vpr to LTR-driven transcription. Specifically, Vpr has been shown to recruit the ubiquitous co-activator p300/CBP to the LTR (Felzien, 1998). CBP and p300 are redundant transcriptional coactivators with intrinsic histone acetyl-transferase (HAT) activity (Kalkhoven, 2004). Acetylation of histones through HAT activity of p300/CBP promotes chromosomal decompression, thus exposing chromosomal regions for transcription. Recruitment of p300/CBP may allow the HIV proteins to remain preferentially transcribed in infected host cells by maintaining the chromosomal region containing the integrated pro-viral DNA in a state of transcriptional activation. This theory is supported by the binding to and recruitment of two general cellular transcription factors, TFIIB (Agostini, 1996) and TFIIH (Kino, 2002), by Vpr. TFIIB and TFIIH are part of the core elongation complex binding to RNA Polymerase II and controlling eukaryotic transcription (reviewed in Warren, 2002). Recruitment of general transcription factors of this class may support an increased frequency of RNA Polymerase II bound to the LTR/proviral DNA.

Recently, evidence has also been published suggesting that Vpr may directly bind DNA, acting as a transcription factor itself. The LTR contains two sites for binding transcription factors known as CCAAT enhancer binding proteins (C/EBP). Hogan et. al. (2003) used EMSA to show that purified, recombinant Vpr directly binds to each of these sites. Further work from that group went on to show that the affinity of Vpr for these sites, which differed between virus strains, correlated with HIV dementia (Burdo, 2004). Further support for these studies comes from another group, which had previously shown that Vpr is capable of binding nucleic acids through a leucine zipper motif (Wang, 1996, Zhang, 1998). Thus, together, through transcription

factor and co-activator binding and recruitment and potentially direct DNA binding, Vpr has consistently been shown to induce transactivation of the HIV-1 LTR. The level of transactivation has been postulated to be ≥4-fold (Wang, 1995, Philippon, 1999, Sawaya, 1999). That transactivation by Vpr occurs in the absence of Tat may play an important role in early expression of viral genes (Hrimech, 1999). Vpr has, in fact, been shown to induce transactivation from unintegrated viral genome (Poon, 2003). Upon the appearance of Tat, Vpr and Tat have an additive effect on viral gene transcription (Sawaya, 2000). Vpr may therefore play a role in viral gene transcription throughout the HIV life-cycle, both alone and in concert with Tat.

Cell Cycle Arrest

Another well described function of Vpr is its ability to induce cell cycle arrest. Vpr has been shown to halt proliferation and induce cell cycle arrest at the G2/M phase (Rogel, 1995, Jowett, 1995). The DNA analyses used to measure cell cycle arrest cannot discern between G2 and M phase cells, but it is generally accepted that the arrest occurs at the transition from G2 to M phase. The transition between G2 and M would be the most logical place for Vpr to act, as this is a major checkpoint prior to cellular entry into mitosis. At this point the cell "checks" whether appropriate growth and DNA synthesis have been completed for progression to mitosis. Cell cycle checkpoints are controlled by cyclin dependent kinases, which act as the gatekeepers. Dephosphorylation of the p34cdc2-cyclin B complex is associated progression at this checkpoint. In the presence of Vpr, p34cdc2-cyclin B is almost completely in an inhibitory, phosphorylated state (He, 1995, Re, 1995). Addition of exogenous phosphatase or dominant active form of p34cdc2-cyclin B released cells from arrest, indicating that Vpr is inducing a signaling change and not necessarily permanent cellular damage. Mutational studies found that a conserved domain at the carboxyl terminal end of Vpr, HFRIGCRHSRIG, containing two H(S/F)RIG motifs, was responsible for cell cycle arrest (Macreadie, 1995, 1996). Specifically, mutation of the G75, S79 or R80 amino acids abrogates the cell cycle arrest function (DiMarzio, 1995, Mahalingham, 1997, Berglez, 1999). Phosphorylation at S79 has been shown to regulate Vpr ability to induce cell cycle arrest (Zhao, 2000). At present, the mechanism for Vpr-mediated cell cycle arrest is not completely understood. The cellular proteins rad24, ppa2, and wee1 kinase have been suggested to play a role (Masuda, 2000, Yuan, 2003). Alternately, induction of the

DNA damage response has also been suggested to play a role in Vpr-mediated cell cycle arrest (Withers-Ward, 1997, Gragerov, 1998). DNA damage would signal the cell to stop proliferating and undergo repairs prior to proceeding to mitosis. Though the mechanism by which Vpr induces cell cycle arrest in not known, it has been shown that this function of Vpr is conserved among primate lentiviruses (Planelles, 1996), indicating an important role in viral replication. In fact, expression of the viral genome has been shown to be optimal when cells are in G2 (Goh, 1998). This may be a nonspecific effect of the increased translational machinery and synthesis occurring during the cell growth in G2 or may be through an as yet undetermined specific effect. Vpr has been shown to induce cell cycle arrest in cells of various lineages, including cancer cells (Mahalingham, 1997, Toy, 2000, Pang, 2001). Elucidation of the mechanism of Vpr-mediated cell cycle arrest may provide novel targets for anti-cancer therapies.

Apoptosis

Exposure of cells to Vpr has also been shown to induce apoptosis. As prolonged cell cycle arrest triggers apoptosis, this effect was originally postulated to be only a byproduct of the Vpr-mediated cell cycle arrest described above. Mutational studies have since shown that induction of apoptosis by Vpr is a specific function which is separable from G2/M arrest (Nishizawa, 2000). Studies of free Vpr protein have indicated that it is capable of triggering apoptosis in uninfected cells (Yasuda, 2001 Bouzar, 2004). This effect is striking in neuronal cultures and it has been hypothesized that Vpr may be a neurotoxin with a role in the neuronal death leading to HIV-associated dementia (HAD) (Patel, 2000, Pomerantz, 2004). *In vivo* Vpr is available as a free non-virion associated protein, carried in non-infectious particles, carried within infectious particles, and synthesized de novo in infected cells (reviewed in Tungaturthi, 2003). Several reservoirs of Vpr would thus be available to affect apoptosis in bystander cells, potentially playing a role in the CD4+ lymphocytes and neuronal cells death associated with HIV disease progression and HAD. In fact, one study detected cell and virion free Vpr in the cerebrospinal fluid of infected patients (Levy, 1994) and our laboratory has shown Vpr to be present within the brain of HAD patients (Wheeler, E, unpublished results).

Presently, though no consensus mechanism for Vpr's apoptotic effects has been reached, several different cellular pathways have been implicated. Initial studies implicated caspase activation as a pathway leading to Vpr-mediated apoptosis (Shostak, 1999, Stewart, 2000).

Caspase activation serves as a convergence point for apoptotic pathways, and caspases are considered the effector signaling molecules of apoptosis. Thus, as the most highly downstream molecules involved in apoptosis, these findings did not explain the mechanism by which Vpr was inducing apoptosis, but did serve to confirm that the death was apoptotic and not necrotic. Studies by Dr. Kroemer's group suggest that the direct effects of Vpr on the mitochondrial membrane may mediate apoptosis (Jacotat, 2000). Vpr induced mitochondrial membrane pore formation through the adenine nucleotide translocator (ANT), which may result in rupture of the mitochondrial outer membrane, a potent inducer of apoptosis (Vieira, 2000). Mutational analysis of Vpr from this group has now revealed that the effects of the Vpr on the mitochondria may be separable from the caspase-dependent apoptotic signaling described above (Roumier, 2002). Studies from other groups, including our own, have also implicated cellular binding partners of Vpr in apoptosis induction. Our group recently found binding of Vpr to a novel cellular protein with anti-apoptotic function, VBARP (Miles, 2005). Interaction of Vpr with the anti-apoptotic cellular protein HAX-1 (Yedavalli, 2005) and the GR have also been suggested as potentially underlying Vpr-mediated apoptosis. It is apparent that this highly important function of Vpr is still poorly understood. Making this subject even more complex is the fact that several studies have also indicated that in some cellular atmospheres, such as T cell receptor engagement, Vpr may play an anti-apoptotic role (Ayyavoo, 1997, Conti, 1998, Fukumori, 1998). These studies present an interesting paradox for Vpr as both a pro- and anti-apoptotic protein. Viral replication would be expected to benefit most from a situation in which Vpr was able to induce apoptosis in uninfected immune cells, thereby evading the immune system, while acting in an anti-apoptotic manner in infected cells, promoting viral replication. At present, the conditions which induce either of these states have not been elucidated. In the study presented here, we attempt to elucidate potential upstream players in Vpr-mediated apoptosis. The array analyses described in Chapter 2 were completed in an effort to determine the cellular genes altered by Vpr. As one of the most potentially important, yet least understood aspects of Vpr biology is apoptosis signaling, we chose to chose from the results of Chapter 2 two genes showed promise as mediators of Vpr-induced apoptosis. The results of the study of those two genes, NHE1 and TNF alpha, and their potential roles in Vpr-mediated apoptosis are described in Chapters 3 and 4.

1.3 STATEMENT OF HYPOTHESIS AND AIMS

Viral protein R of HIV-1 (Vpr) has been shown to increase transactivation of the HIV-1 promoter, or LTR (Cohen[2], 1990, Vanitharani, 2001, Thotala, 2004). Vpr accomplishes this through binding to and utilization of several cellular transcription factors, including; the glucocorticoid receptor (Rafaeli, 1995), Sp1 (Wang, 1995), p300 (Kino, 2002), TFIIB (Agostini, 1996), and potentially others (Bouhamdan, 1996, Goh, 2004, Kino, 2005). The binding of Vpr to these cellular transcription factors and their use in virus replication would be expected to have repercussions in cellular gene expression. Potential effects include downregulation of genes due to transcription factors available in limited quantities being sequestered on the LTR and upregulation due to the formation of novel or not normally favored transcriptomes. The effects of Vpr on total cellular gene expression have not been studied to date. In addition, while the biological effects of Vpr, including induction of apoptosis, have been well documented, mechanism data is incomplete. The studies thus far have mainly detailed potential pathways mediating the late events in Vpr-mediated apoptosis, such as caspase induction (Shostak, 1999, Stewart, 2000) and mitochondrial membrane perturbation (Jacotot, 2001, Roumier, 2002). An analysis of potential early cellular mediators of apoptosis, those beginning the cascade of downstream events such as caspase activation, has not been forthcoming. Thus, in this analysis, we undertook high throughput analyses of cellular gene expression changes mediated by Vpr. **We hypothesize that Vpr induces changes in cellular gene expression and that modulation of specific cellular genes may correlate with known Vpr functions, such as apoptosis.** We addressed this hypothesis through the completion of the following specific aims.

Specific Aim 1: Determine the effects of HIV-1 Vpr on host cellular gene expression.

We undertook high throughput analyses consisting of microarray and antibody array experiments in order to determine potential cellular gene expression changes modulated by Vpr at the mRNA and protein levels. The high throughput data generated were confirmed through the use of real time RT-PCR and western blot.

Specific Aim 2: Determine the effect of Vpr on NHE1 expression in HIV infected cells.

Results attained in Specific Aim 1 indicate that one of the genes showing the largest modulation by Vpr is the Na+/H+ Exchanger, isoform 1. In this Aim, we confirmed that NHE1 is downregulated by Vpr in HIV infected cells. The cellular effects of NHE1 modulation were explored, with the goal of determining whether NHE1 modulation may contribute to Vpr-mediated induction of apoptosis of host cells.

Specific Aim 3: Determine the effect of Vpr on TNF alpha regulation in HIV-1 infected cells.

Results of the analysis in Specific Aim 1 implicated TNF alpha as possessing a promoter similar to other genes regulated by Vpr. As a "secreted factor", most likely a cytokine/chemokine, has been suggested to be induced by Vpr, we investigated the ability of Vpr to induce TNF alpha secretion from infected macrophages. A potential role for TNF alpha in apoptosis of uninfected bystander cells was assessed.

1.3.1 Organization of Study and Fulfillment of Specific Aims

Three chapters follow this introduction and present the results attained in this study. The first is Chapter 2, which is included as fulfillment of Specific Aim 1. The main context of Chapter 2 is a manuscript which was published in Biochemical and Biophysical Research Communications and has been reprinted with permission from Elsevier Science (Section 6.0). This manuscript details the initial cDNA microarray experiments proposed in Specific Aim 1, as well as the follow-up quantitative RT-PCR and western blot experiments to verify microarray results. The results of the microarray analysis were exciting and were the first published evidence that Vpr affected transcription of host cellular genes. Previous studies had been focused mainly on the affect of Vpr on the LTR or on single artificial heterologous promoter

constructs. Several distinct classes of host cellular genes were now found to be differentially regulated by Vpr. These results raised a new question of whether the changes seen at the mRNA level were robust enough to induce changes in cellular signaling which may lead to the known "effects" of Vpr on the cell. A new technology at the time was an "antibody array", which had just been released from BD Biosciences/Clontech. The array consisted of antibodies to particular proteins attached in clusters on the slide. Similar to the microarray technology, we could now compare the relative amount of cellular proteins in host cells infected with HIV with or without expression of *vpr*. Due to the much larger size of antibodies, as compared to oligonucleotides, the antibody array was limited to less than $1/14^{th}$ the size of the microarray. Given that and the small size of the cDNA microarrays available at the time, most of the genes on the antibody array did not overlap with those on the microarray. Yet, this was inconsequential in answering our question, which was whether Vpr could induce changes in expression of host cellular signaling proteins. If the results were interesting, they could be correlated with transcriptional control by "back-checking" the antibody array results with quantitative RT-PCR, in the same way that we had "forward-checked" the array results with western blot. In fact, the results were interesting, as 18 of the 384 proteins being tested on the protein array (approximately 21%) were differentially regulated by Vpr at a ratio ≥ 2. This showed that Vpr was inducing significant changes in signaling in the signaling proteins in the cell.

In Chapter 3, I went on to "back-check" the expression of one of these highly regulated proteins, NHE1, as well as determine the effects of this change in the host cell. Prior to this, though, I was faced with an immense amount of high-throughput data to try and make sense of. It always bothered me a bit that Vpr could be inducing changes in so many different cellular proteins. Vpr is a very small protein, 14kD, and as a viral protein, I would not expect it to be present in the cell at staggering amounts. So, there had to be a common thread between all of these differentially regulated genes. I then painstakingly mapped the promoters of several of the most highly regulated genes and found some common threads, which are described at the end of Chapter 2. As new software packages for analyzing high throughout data sets are now becoming available and more user-friendly, it may prove prudent to reanalyze much the data generated here in these "pathway analysis" packages.

Another interesting feature of array analyses is that they often produce many leads. One such lead that our laboratory explored further was the effect of Vpr transcriptional regulation on

host immune dysregulation. The microarray and protein arrays showed several immunoregulatory genes to be differentially regulated by Vpr (See Table 1 and Table 2). The differential regulation of those genes was used as a basis to look further at the effect of Vpr on immunomodulatory molecules. Those studies became part of Dr. Biswanath Majumder's work in our laboratory on the immune regulation induced in host monocytes by Vpr. In support of his work, I found the transcriptional downregulation of several immunoregulatory molecules, CD83, CD80, and CD86, also found the upregulation of TNF alpha by Vpr (Majumder, 2005, 2006). As a byproduct of the original microarray analyses, this gene was explored further for its regulation by Vpr at the transcriptional level in Chapter 4. Thus, both Chapter 3 and Chapter 4 came directly and indirectly, respectively, from the original array analyses presented here.

Chapter 3 is now presented in fulfillment of Specific Aim 2 and Chapter 4 is presented in fulfillments of Specific Aim 3. The body of Chapter 3, concerning regulation of the cellular protein NHE1 by Vpr, is mainly composed of a manuscript being prepared for submission. The manuscript includes the antibody array analyses, which are presented here instead in Chapter 2, to which their logic belongs. In order to aid in the flow of this manuscript, the antibody array analyses were placed in Chapter 2 and removed from the manuscript in Chapter 3. Thus, a new Introduction and Discussion were also written for that Chapter. When the manuscript appears in print, it will be slightly altered, then, from what is presented here. Chapter 4 has not been considered for publication yet, as larger studies are needed to verify the percentages attained. Both Chapter 3 and Chapter 4 are significant in that each takes a gene found to be differentially regulated by Vpr and explored the consequences of this regulation in the host cell. Both genes were found to be related to function of Vpr described in the Introduction above, induction of apoptosis in infected and bystander cells. These results now link transcriptional regulation of host cellular genes to known functions of Vpr, providing two novel pathways for further study in HIV disease progression and its potential therapeutic target points.

2.0 CHAPTER 2: DIFFERENTIAL REGULATION OF HOST CELLULAR GENES BY HIV-1 VIRAL PROTEIN R (VPR): CDNA MICROARRAY ANALYSIS USING ISOGENIC VIRUS

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Janket ML, Manickam P, Majumder B, Thotala DK, Wagner M, Schafer E, Collman R, Srinivasan A, and Ayyavoo V.

Differential regulation of host cellular genes by HIV-1 Viral protein R (Vpr): cDNA microarray analysis using isogenic virus.

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2.1 ABSTRACT

HIV-1 Vpr is a protein with multiple functions. It has been suggested that such pleiotropic effects by a viral protein may be mediated by its association with viral and cellular proteins or through modulation of expression of specific cellular genes. To address this, we used cDNA microarray techniques to analyze the regulation of a panel of host cellular genes by HIV-1 Vpr using isogenic HIV-1 either with or without Vpr expression. Results indicate that Vpr downregulated the expression of genes involved in cell cycle/proliferation regulation, DNA repair, tumor antigens, and immune activation factors, and upregulated many ribosomal and structural proteins. These results for the first time reveal the involvement of several potential cellular genes, which may be useful, both for understanding Vpr functions and for the development of therapeutics targeting the Vpr molecule.

2.2 INTRODUCTION

HIV-1 infects and destroys several target cell types including cells of the immune and nervous systems leading to overt diseases (Levy 1998). It has been hypothesized that the cytopathic effects resulting from viral infection may be partly due to the interaction between virally encoded proteins and host cell proteins through either direct and/or indirect mechanisms. In addition to the viral proteins directly affecting the infected target cells, killing of bystander cells may also be initiated through the interactions of the infected and uninfected cells or through the release of viral proteins from the infected cells (Levy 1998, Herbein, 1998). The cytopathic effects noted with HIV-1 infection have been linked to its structural and accessory gene products (Ayyavoo 1997, Azad, 2000, Haughey, 2002, Rasola, 2001).

HIV-1 *vpr* gene encodes a protein of 96 amino acids with a predicted molecular weight of 15 kDa, and is relatively conserved in HIV and Simian Immunodeficiency Virus (SIV) (Cohen 1990). Studies by several investigators have shown that Vpr is a pleiotropic protein, with diverse

functions including cell cycle arrest at the G2/M phase, apoptosis, nuclear localization, nuclear import of the preintegration complex, transcriptional activation and interactions with viral and cellular proteins (Goh, 1999, Heinzinger, 1994, Levy, 1993, Vanitharani 2001). Interestingly, there are multiple sources of Vpr, such as intracellular (cell-associated Vpr), intravirion (virion-associated Vpr) and free Vpr (cell-free and virus-free) available in infected individuals to mediate these functions (Tungaturthi, 2003). There are several lines of evidence in support of the involvement of the multiple forms of Vpr in pathogenic events and also in the regulation of HIV-1 replication.

Despite a vast number of studies reported on Vpr, the mechanism by which this protein is able to bring about its biological effects is not clear. To gain a better understanding of the mechanisms underlying Vpr functions, we have analyzed the effects of Vpr on the expression of host cellular genes with the notion that such an analysis may provide information about the involvement of genes not yet identified through biochemical approaches. For this purpose, we have employed cDNA microarray analyses of PBMCs infected with isogenic HIV-1 either with or without the expression of Vpr. The results demonstrated that Vpr downregulated the expression of a number of cell cycle, tumor-associated cellular genes and a number of genes involved in immune activation events, correlating with the previously described functions of Vpr. Vpr upregulated a number of ribosomal proteins and structural proteins. Together, these results suggest that HIV-1 Vpr influences virus replication and cytopathic effects through regulation of several host cellular genes independent of its cell cycle arrest function.

2.3 MATERIALS AND METHODS

Virus Infection

HIV-1 proviral DNAs 89.6wt (wild type with an intact *vpr* gene), and 89.6*vpr*- (containing a frameshift mutation in Vpr coding sequences to eliminate its expression) were constructed as described (Balliet 1994). PHA-stimulated PBMCs (10 x107) from normal healthy human individuals were infected with viruses containing 100 ng p24 antigen equivalents of 89.6wt or 89.6*vpr*- virus stocks as described (Balliet, 1994). Seventy-two hours post infection total RNA was isolated and used for microarray hybridizations.

cDNA preparations and Array Hybridization.

Preparation of probes, cDNA array hybridization, scanning of phosphorimages, and data analyses were all performed as described previously (Bosetti 2002). All hybridizations were performed using a GF211 'known genes' Genefilter microarray (Research Genetics). This array consists of 4132 individual elements, each representing a known human gene. Changes in expression levels were calculated using normalized intensities and given as ratios. Expression ratios J2.0 in duplicate assays were considered significant and are reported here.

Quantitative RT-PCR using Taqman.

To confirm the expression ratios seen with microarray, the mRNA levels of selected genes were quantified by real-time RT-PCR. Primers were designed across splice junctions where it was possible to eliminate the amplification of potential contaminating genomic DNA and were tested by standard PCR methods for genomic DNA amplification prior to use. Probes used in this study were 5' 6Fam labeled 3' TAMRA. Real-time PCR was run using the ABI Prism 7700 Sequence Detection system. Data were analyzed via Sequence Detector v1.0 software. All values were normalized to the endogenous control GAPDH. Assays were repeated in triplicate and average threshold cycle values were used to determine relative concentration differences based on the ΔΔCT method described in the manufacturer's protocol, User Bulletin #2 (ABI, 1997).

Protein expression by Immunoblotting and Flow cytometry.

Normal human PBMCs were cultured and infected as described above. Whole cell lysates were prepared and 20μg equivalents of protein were separated by SDS-PAGE, transferred to Nitrocellulose membrane (Bio-Rad) and probed with rabbit polyclonal anti-HSF-1 (Affinity Bioreagents) or mouse monoclonal α-tubulin (NeoMarkers) for 1 hr at room temperature, and then washed and incubated with goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Caltag). The membrane was developed using an ECL enhanced chemiluminescence kit (Amersham Biosciences). For flow cytometry experiments, PHA-stimulated PBMCs were infected with 89.6wt or 89.6*vpr*- as described. Forty-eight hours post infection, cells were stained

with mouse monoclonal anti-CD81-FITC (Caltag) and analyzed by flow cytometry (Beckman Coulter).

2.4 RESULTS AND DISCUSSION

DNA array analysis of peripheral blood mononuclear cell responses to infection by HIV-1 either with or without Vpr expression.

The main goal of the study presented here is to analyze the influence of Vpr on the expression of a panel of host cellular genes in an effort to understand the basis of the biological effects of HIV-1 Vpr. Although plasmid or viral vectors containing the gene of interest under a heterologous promoter have previously been used in different studies, it is clear that the level of protein directed by such vectors may not truly reflect the amount expressed in the context of the authentic virus infection. This has prompted us to employ isogenic virus with and without Vpr expression as well as peripheral blood mononuclear cells (PBMCs) to mirror the infection by HIV-1 in vivo. PHA-stimulated PBMCs were infected with isogenic HIV-1 89.6wt or 89.6*vpr*- viruses. Based on the viral inoculum used, >60 % of cells showed evidence of infection as determined by immunoflourescence assay for the structural protein Gag (data not shown). The pattern of cellular messenger RNA from cells infected with the 89.6wt was compared to the cells infected with 89.6*vpr*-. Each experiment was carried out in duplicate (using PBMCs from two separate donors) and the results presented here represent the genes that were positive in both screenings. Using DNA microarrays containing 4132 full-length cDNA clones, we have identified approximately 71 genes that were differentially regulated by HIV-1 Vpr by 2 fold or higher (Table 1 & 2). The genes downregulated were cell cycle proteins, tumor-associated receptors, stress induced proteins and immune activation markers. In particular, a number of molecules such as Endoglin, hormone receptor, and heat shock associated proteins were significantly regulated by Vpr (> 6 to 20 fold). The observed downregulation of cell cycle genes and tumor antigens ranged from ratios of 1.8 to 4.0.

The major class of genes upregulated were ribosomal proteins and structural genes. These results are in agreement with the studies by Geiss et al. (2000), Vahey et al. (2002) and van't

Wout et al. (2003) who also showed a similar upregulation of ribosomal proteins as a consequence of synthesis of viral proteins in HIV-1 infected PBMCs compared to uninfected controls. Our results further support the effect of Vpr on viral gene expression. It is also important to note that in addition to the regulation of heat shock proteins due to overall HIV-1 infection, presence of Vpr increased the expression of HSF-1 (Heat shock factor 1) dramatically (>14 fold). These observations are in accordance with the known information on the role of DnaJ-like proteins in GR: hsp90 assembly (Dittmar, 1998, Zou, 1998).

The presence of DNAJ (hsp40) is required for GR-hsp90 heterocomplex formation and sequestration of GR in the cytoplasm (Dittmar, 1998). Conversely, upon activation HSF-1 releases GR from the hsp90 complex and translocates GR into the nucleus. These data suggest that Vpr might either directly regulate the transcription of the genes or act indirectly through the GR coactivator pathway which in turn regulates the feedback mechanism of HSP-HSF transcription. Furthermore, these studies support and provide a potential mechanism to explain the role of Vpr as a GR coactivator (Vanitharani, 2001, Kino, 1999, Sherman, 2000).

Similarly, N10 also known as TR3 and nur77 is an orphan member of the steroid/thyroid/retinoid receptor family that is known to regulate gene expression (Mangelsdorf, 1995). Expression of TR3 is increased by several mitogenic stimuli (Williams, 1993, Kolluri, 2003). Our results indicate that expression of N10 was drastically reduced (ratio of 13.1) in cells infected with *vpr* containing virus compared to *vpr* deleted virus. These results further support that the Vpr-induced inhibition of cell proliferation in PHA-stimulated primary lymphocytes could be through N10.

Table 1: Genes Downregulated by HIV-1 Vpr

Accession #	Gene	Ratio
Receptors		
AA486556	CD81 antigen	2.8
AA630094	Human mRNA for P2Y6 receptor	3.6
R56604	Cholinergic receptor, nicotinic, alpha polypeptide 4	3.1
AA446928	Erbb2- Receptor protein tyrosine kinase precursor	3.6
AA446108	Endoglin (Osler-Rendu-Weber syndrome 1)	6.2
Adhesion		
AA485668	Integrin beta 4 subunit	3.0
AA055979	Integrin, alpha 7B	3.1
AA448400	Human plectin (PLEC1) mRNA	3.7
AA159577	Mucin 5, subtype B	2.6
Transcription/Translation factors		
AA026644	Transcription factor 3	3.2
AA454609	Human forkhead transcription factor HFH-4	3.1
Cell cycle		
H73724	Cyclin dependent kinase 6	1.5
AA877595	Cyclin dependent kinase inhibitor 2A	2.0
AA486233	G1 to S phase transition 1	2.1
AA458870	Human CDC37 homolog	2.1
H15456	Calpain 1, Large	3.7
AA630094	Human siah binding protein 1 (SiahBP1) mRNA	3.2
Tumor associated/oncogenes		
AA291556	Human ras inhibitor mRNA, 3' end	3.3
R44982	Homo sapiens putative tumor suppressor protein unspliced form	1.8
R53541	H.sapien mRNA for melanoma associated chondroitin sulfate proteoglycan	3.1
W80632	Human BRCA2 region	1.5
Immune function		
N30372	Interferon regulatory factor 5	2.6
R50953	Human B lymphocyte serine/threonine protein kinase mRNA	2.6
AA443090	Human interferon regulatory factor 7 mRNA	2.7
AA488406	Human RNA for pre-pro-megakaryocyte potentiating factor	2.9
N90281	Human B7 mRNA	2.1
N35070	H. sapiens TWEAK mRNA	3.4
Signal transduction		
N68166	Human signal transducing guanine nucleotide binding regulatory G protein beta	3.0
N54551	Human serine-threonine phosphatase (PP5) mRNA	2.3
Miscellaneous		
AA598861	H.sapiens mRNA for a cell surface protein	2.9
AA683085	High mobility group (nonhistone chromosomal) protein 1	2.9
H41489	Adaptin, beta 1 (beta prime)	3.4
H45449	Arylsulfatase A	3.3
AA703449	Human Meis-1 related protein 2	3.7
H45618	Human apm1 mRNA for GS3109 (collagen like factor)	19.3
N94487	Hormone receptor (growth factor inducible nuclear protein N10)	13.1
AA872020	H. sapiens prostasin mRNA	3.0
AA455298	DNAJ protein homolog HSJ1	20.1

Table 2: Genes Upregulated by HIV-1 Vpr

Accession #	Gene	Ratio
Receptors		
AA629897	Laminin receptor (2H5 epitope)	2.5
Signal transduction		
AA487575	Human Snk interacting protein 2-28	2.8
AA454702	Cellular retinoic acid binding protein	2.2
H97140	Human protein tyrosine phosphatase	2.6
Gene Expression		
AA047260	H. sapiens histone H2A, mRNA	2.7
AA868008	H.sapiens H4/g gene for H4 histone	2.4
Translation		
AA775364	60S Ribosomal protein L30	2.4
AA669359	Ribosomal protein L44	2.4
AA599178	Ribosomal protein L27a	2.6
AA633768	60S Ribosomal protein L24	2.8
AA634008	40S Ribosomal protein S23	2.4
AA683050	40S Ribosomal protein S8	2.6
AA464743	Ribosomal protein L21	2.7
AA873351	Ribosomal protein L35a	3.7
AA083485	Ribosomal protein L19	2.7
AA888182	Ribosomal protein S4, X linked	3.0
AA411343	Ribosomal protein S29	2.5
AA668301	Ribosomal protein S16	3.0
AA629641	Ribosomal protein S13	3.5
AA680244	H.sapiens mRNA for ribosomal protein L11	2.5
R37286	Human hnRNP core protein A1	2.0
Metabolism		
H16958	Human glyceraldehydes 3 phosphate dehydrogenase	2.5
Structural		
AA670408	Beta 2 microglobulin precursor	2.8
R44290	Human cytoplasmic beta-actin gene	3.4
AA479882	Keratin 10	2.4
R54968	Alpha-1 type XVI collagen	2.1
Proliferation		
AA442991	Prothymosin alpha	2.2
AA634103	Human thymosin beta-4	2.8
Stress response		
AA449119	Heat shock factor protein 1	14.4
Immune function		
AA464246	Major histocompatibility complex, class 1, C	2.7
W73144	Lymphocyte cytosolic protein 1 (L-plastin)	3.1
Miscellaneous		
T67270	Ubiquitin cytochrome C reductase complex	2.8
AA862813	Cytochrome C oxidase polypeptide VIII	2.5
H67086	H. sapiens TEB4 protein mRNA	2.0
AA625632	Ubiquitin A-52 residue ribosomal protein fusion product 1	2.9
AA410517	Cytoplasmic antiproteinase	2.8
AA430524	Angiotensin converting enzyme precursor	2.0

Analysis of the expression of host cellular genes by real-time RT-PCR using Vpr in the context of virus.

Real-time quantitative RT-PCR (Taqman) was used to corroborate the values obtained from microarray results. In carrying out this assay we chose a small panel of genes encompassing different families as well as varying ratios. The results shown in Figure 2 A-D are representative of data generated from each specific gene. All assays were run in triplicate, and reported threshold values represent the mean of three independent experiments, with standard deviations as noted (to determine expression ratios, the threshold cycle was used in accordance with the ΔΔCT method designed by the manufacturer as a method to calculate relative gene expression). Taqman results were found to validate the microarray data. Representative data for the four genes shown here reflect the differences observed in the microarray results. For instance, CD81 with a 2.8 fold reduction in the presence of Vpr seen by microarray analysis showed a 2.01 + 0.1 fold reduction via real time RT-PCR analysis (Figure 2A). Similarly, the microarray ratio (13.1) seen for nuclear protein N10 was in correlation with the 12.10 + 0.1 fold decrease observed by real time RT-PCR (Figure 2B). HSF-1 was determined by microarray to be upregulated by a ratio of 14.4 and real time RT-PCR showed a consistent upregulation of 24.61 + 0.6 (Figure 1C). Additionally, another heat shock associated protein, DnaJ homolog also showed similar results (data not shown). GAPDH and B2M (beta-2-microglobulin) were used as controls for the RNA content in all our experiments and a representative figure from one experiment is shown in Figure 2D. It should also be noted that the microarray experiments described above utilized RNA derived from cells of two different normal healthy donors. Results from these, as well as from three different donors used for RT-PCR experiments, were consistent in regard to both regulated genes and their respective ratios, suggesting that the Vpr-mediated effect on global gene regulation is well conserved.

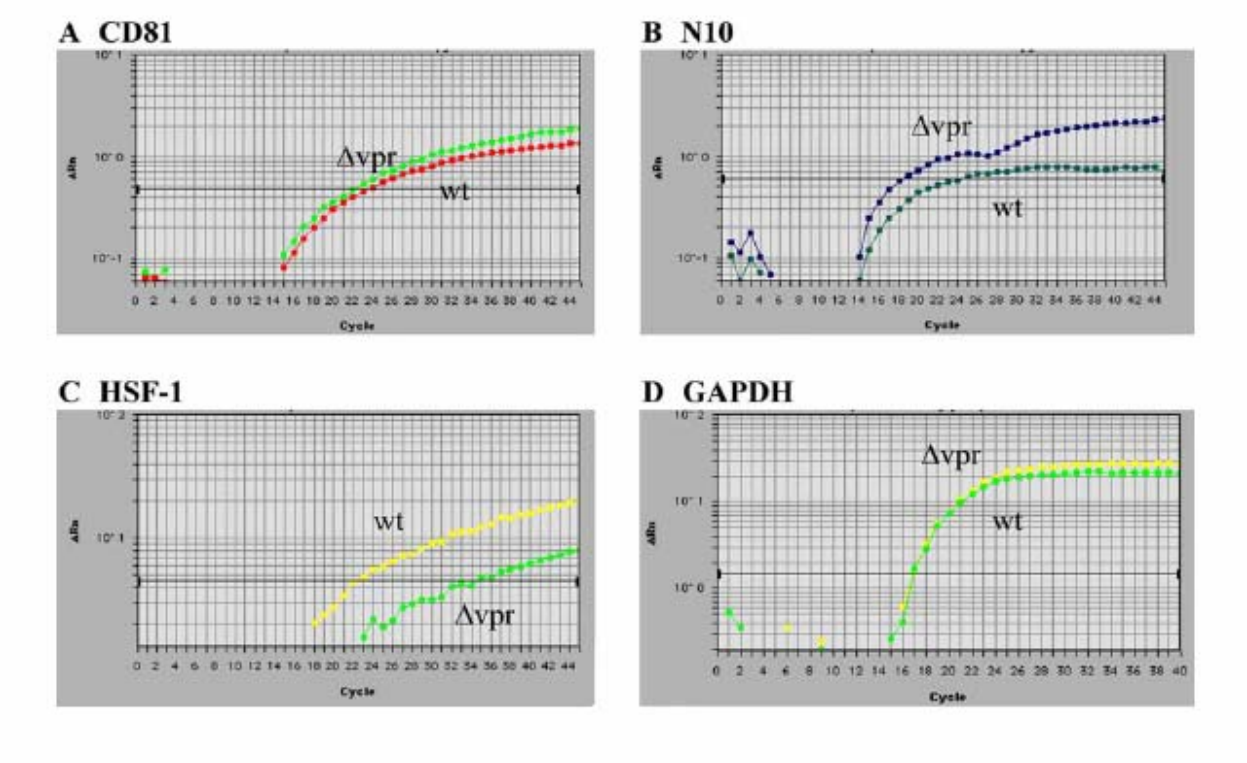

Figure 2: Real-time RT-PCR analysis to confirm microarray results.

RNA was isolated from PBMCs infected with HIV-1 89.6wt or 89.6vpr-. Gene specific primers and probes were used to amplify 150–200 bp of each target gene. Figure represents one of the triplicate runs using RNA isolated from different donors. (A) CD81 (TAPA); (B) N10, nuclear antigen N10; (C) HSF-1, heat shock transcription factor 1; and (D) GAPDH, glyceraldehyde-3 phosphate dehydrogenase, as standard. wt, PBMCs infected with 89.6wt; Dvpr, PBMCs infected with 89:6vpr-.

Next, we performed additional experiments to determine whether the gene expression profiles seen here could also be mediated by Vpr in the absence of other viral proteins. Using RNA derived from HeLa cells transfected with pcDNA3.1vpr plasmid or control pcDNA3.1 plasmid we assessed the differential regulation of CD81, N10 and HSF-1 (Figure 3). Results indicate that Vpr in the absence of other proteins regulated the expression of the tested genes similar to that associated with virus infection. For instance, CD81 and N10 were downregulated similar to that found in the microarray and virus treated samples, at ratios of 1.98 and 5.52, respectively compared to a corresponding vector transfected control. Vpr transfection also increased the expression of HSF1 at a ratio of 24.61 compared to vector transfected controls. The ability of Vpr to alter expression of heat shock factor 1, the principle transcription factor controlling the expression of cellular heat shock proteins, indicates a potential role for Vpr in

modulating the stress response in infected cells. Previous work by other laboratories has shown that the level of stress response proteins is specifically altered in HIV infected cells, though a specific viral product responsible has not been determined. The ability of Vpr, in the absence of other viral proteins, to alter the expression of HSF-1 suggests that this protein may play a role in this phenomenon. We are currently exploring this further and preliminary results indicate that Vpr is able to alter the level of HSF-1 responsive genes, including HSP70 (unpublished data). The altered expression of HSF-1 by Vpr, as observed in this study, could potentially affect a wide array of proteins controlled through heat shock responsive promoters, including those involved in signal transduction mechanisms.

Confirmation of protein expression by western blot and FACS analysis.

To further confirm that the changes seen at the RNA level were consistent through to the protein level, we utilized western blot and flow cytometry to determine the levels of the two proteins HSF-1 and CD81 (TAPA). Briefly, PBMCs infected with 89.6wt or 89.6*vpr*- viruses were assessed for CD81 and HSF-1 expression using specific antibodies. We chose to look at HSF-1 due to its significant change in expression (14-fold increase) and stated relevance to Vpr/GR interaction (Vanitharani, 2001, Kino, 1999). Protein expression measured by western blot using HSF-1 specific antibody is presented in Figure 4. Results from this analysis indicate that the presence of Vpr increased the expression level of HSF-1 by two-fold compared to cells infected with 89.6*vpr*- virus. These results are in concurrence with the upregulation seen by both microarray and real time RT-PCR. Though the ratios of change do not correlate between RNA level and protein level, this was expected due to the difference in sensitivity of the two assays (real time RT-PCR versus western blot). Such a difference in ratios has been found by other groups when comparing microarray or real time RTPCR results to protein levels (Li, 2002). Additionally the expression of HSF-1 was immediate early and late, that could correspond to the presence of Vpr early as virion-associated as well as during de novo synthesis upon infection. To further confirm this reasoning we tested a protein with a smaller ratio of RNA expression, CD81 (2.1 fold). Results from FACS analysis (data not shown) of surface CD81 levels indicated no differences between 89.6wt and 89.6*vpr*- infected PBMCs suggesting that a significant fold difference at the RNA level might be necessary to see the differences at the protein level.

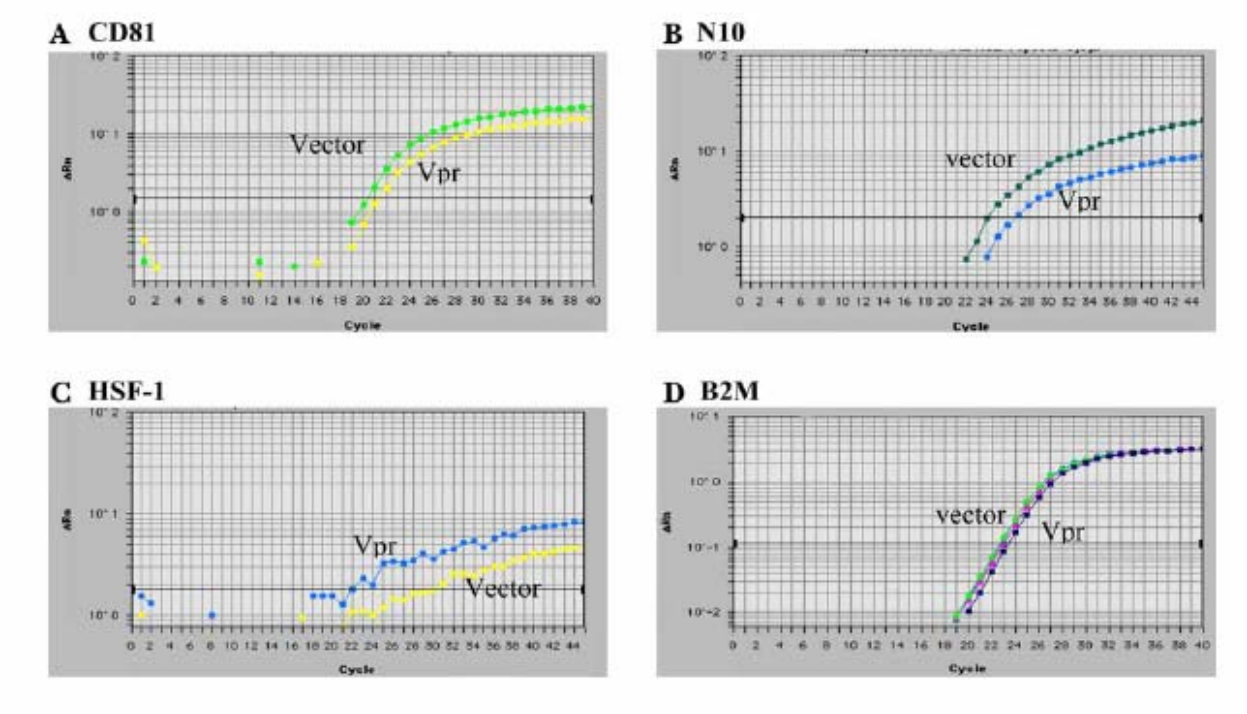

Figure 3: Real-time RT-PCR analysis of Vpr transfected cells.

HeLa cells were transfected with pcDNA3.1-vpr (Vpr) or pcDNA3.1 control plasmid (vector). RNA was isolated from cells 48 h post-transfection. Gene specific primers and probes were used to determine the expression of the genes indicated below. Threshold cycle CT values were calculated using Sequence Detector software. (A) CD81 (TAPA); (B) N10, nuclear antigen N10; (C) HSF-1, heat shock transcription factor 1; and (D) B2M, b-2-microglobulin, as standard.

Together these results indicate that Vpr regulates a number of genes involved in cell cycle, DNA repair and immune activation or cell signaling. Though these results stem from an in vitro system, we feel that the use of Vpr in the context of virus in human PBMCs is the best available system to begin preliminary studies of the effects of Vpr on gene expression during HIV infection. Also supporting this data is the fact that all the results presented here have been corroborated between two separate experiments as well as different donor PBMCs. However, at this point the extent of these effects and the manner in which they may be interrelated in vivo is not known. Detailed studies addressing the direct and indirect effects of Vpr-mediated cell cycle arrest and transactivation functions are needed to gain a better understanding of Vpr's contribution to HIV pathogenesis. The use of gene expression analyses in the identification of the genes involved in Vpr-mediated immunopathogenesis, cell cycle arrest and apoptosis may provide leads as the to potential pathways to target in the development of antiviral drugs

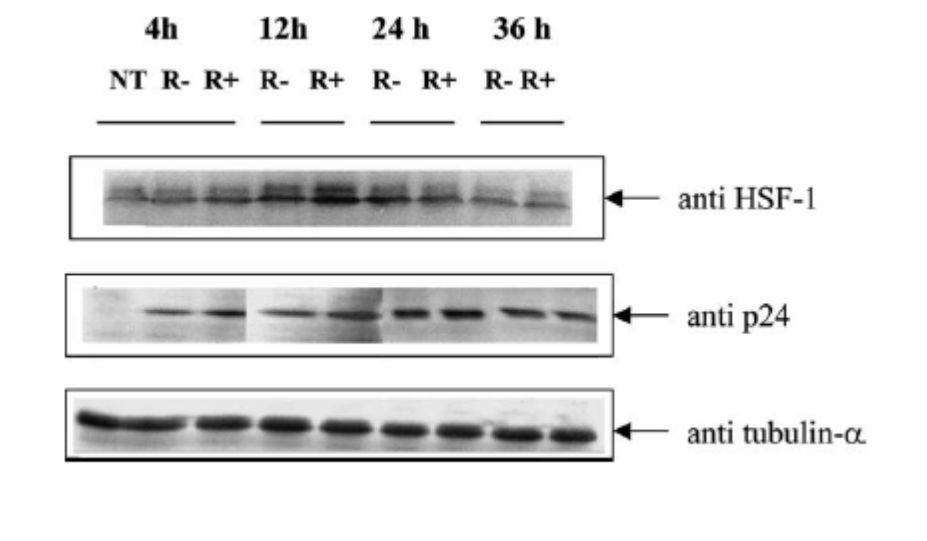

Figure 4: Expression of HSF-1 protein by Western blot analysis.

Equal amount of protein from PHA-stimulated PBMCs infected with virus for the indicated times was resolved by SDS–PAGE and immunoblotted with anti-HSF-1, anti-p24 or anti-tubulin antibody. NT, uninfected control; R), lysate from 89.6vpr- virus-infected cells; and R+, lysate from 89.6wt virus-infected cells.

targeting Vpr. Given the dependence of macrophage infection by HIV-1 on Vpr, such drugs would provide a strategy to prevent macrophage infection. It can be postulated that such effects might be useful in depleting the viral reservoir and reversing the immunosuppressive effect of Vpr during infection. Additionally, the specific downregulation by Vpr of tumor specific gene expression may also provide opportunities in cancer therapy. Identifying the pathways by which Vpr is able to cause specific regulation of both cell cycle and tumor gene expression may open up new targets for cancer drug development.

2.5 ACKNOWLEDGMENT

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2.6 ADDITIONAL EXPERIMENTS OF SPECIFIC AIM ONE

As described in the Statement of Hypothesis and Specific Aims section of this manuscript, two additional analyses on the effects of Vpr on cellular genes expression were carried out as part of Specific Aim 1 following the completion of the preceding manuscript. These analyses, antibody array and promoter mapping, are included below.

2.6.1 Antibody Array

Materials and Methods:

In an effort to detect changes induced by Vpr on host cellular protein expression and cellular signaling, we performed antibody array using Ab Microarray 380 slides purchased from BD Biosciences, USA, following the manufacturer's protocol. Briefly, normal human PBMCs were infected with 89.6wt and 89.6*vpr*- viruses as described in Section 2.3. Cell lysate preparation, protein labeling with Cy5 and Cy3, hybridization, and scanning were performed according to the manufacturer's protocol. To account for differences in the binding of the Cy5 and Cy3 dyes, two slides are provided by the manufacturer for reverse color labeling; wt/Cy5 and vpr-/Cy3 were mixed and added to slide 1, and wt/Cy3 and vpr-/Cy5 were mixed and added to slide 2. Labeled proteins were added to the slides and hybridization was carried out for 30 minutes at room temperature (25-27°C). Data analysis was done using GenePixPro software and expression ratios were calculated using the Microsoft Excel template provided by the manufacturer. The Internally Normalized Ratio (INR) corrects for differences in the reverse color labeling and is calculated as follows: INR =√(Ratio 1/ Ratio 2), where Ratio 1 is sample A Cy5 / sample B Cy3 and Ratio 2 is sample B Cy5 / sample A Cy3. An INR >1.3 or <0.7 is considered by the manufacturer to be significant.

Results and Discussion:

Previous work from our laboratory indicated that HIV-1 Vpr induced changes in host cellular transcription (Janket, 2004). Based upon these findings, we rationalized that the changes

at the transcriptional level could directly alter the protein expression of the same genes and/or indirectly alter the expression of other proteins that are involved either in downstream or upstream events. In order to determine the cellular pathways that are potentially affected by Vpr, we performed an antibody array, which consisted of antibodies corresponding to 378 human cellular proteins spotted duplicate high density spots on a glass slide. Cell lysates prepared from PBMCs infected with 89.6wt or 89.6*vpr*- viruses were subjected to the array procedure described above and the results are presented in Table 3. Average R/R and average INR were calculated using internal normalization controls to improve the stringency and quality of the data. Average R/R represents the raw ratio of the wt versus the vpr- virus infected cells, averaged over both sets of data (each sample was labeled and run twice). INR represents the internally normalized ratio, which was normalized to correct for differences in the way proteins bound the dyes used here, Cy3 and Cy5. In order to generate the INR, samples were labeled twice, once with Cy3 and once with Cy5, and the Cy3/Cy5 ratio and the Cy5/Cy3 ratio of each sample was used to correct the sample ratio. In this report we considered only INR values > 2.00 and <0.6 for upregulated and downregulated protein expression, respectively, to increase the confidence level. Results in Table 3 indicate that of the 378 proteins tested, 16 proteins showed upregulation at a ratio greater than 2 fold. Conversely, only four proteins of the 378 tested showed downregulation at a ratio of >0.6. Among the upregulated and downregulated proteins, diverse signaling families were represented. The most highly regulated upregulated protein, synaptotagmin functions as a calcium sensor in synaptic neurotransmitter release (Sorensen, 2003). Expression of synaptotagmin mRNA (as assessed by real time RT-PCR) was found to be higher in neuronal culture than in PBMCs, consistent with its known functions. Thus, the potential role of upregulation of this protein is being explored in an alternate project within our laboratory focusing on the neurotoxic properties of Vpr. The most highly downregulated gene was PARP-1, poly(ADP-ribose) polymerase, which has been suggested to play a role in HIV integration (Ha 2001) Real time RT-PCR did not show downregulation of this gene by Vpr at the transcriptional level. The downregulation of PARP protein may therefore be at a step post-transcription. Also, PARP-1 is cleaved by caspase 3 during induction of apoptosis and thus the noted downregulation may have been due to increased apoptosis in Vpr-containing cells. Studies are ongoing in our laboratory to further determine whether downregulation of this protein has a role in Vpr function. NHE1, the sodium hydrogen exchanger isoform 1, was the second largest ratio of

downregulation, at an INR of 0.41. NHE1 was chosen for further study in Specific Aim Two (Chapter 3) based on its described role in cell cycle arrest and apoptosis, two functions associated with Vpr.

Table 3: Effects of HIV-1 Vpr on host cellular protein expression

Upregulated			
Ab-Ag	**Average R/R**	**Average INR**	**Accession #**
Synaptotagmin	64.91327323	8.056877387	P21579
PKA rIa	46.35502215	6.808452258	P10644
V-1/myotrophin	9.311514557	3.051477438	P80144
p300	8.337555007	2.887482469	Q09472
IRS-1	8.225115562	2.867946227	P35568
FIN13	7.375113536	2.715716026	Q92913
DNA Polymerase e	6.309965775	2.511964525	Q07864
eIF-4g	6.220214528	2.494035791	P06730
CRP1	5.542258448	2.354200172	P21291
p19 skp1	5.36669334	2.316612471	P34991
ZFP-37	4.748656696	2.179141275	Q9Y6Q3
E2F-2	4.624880821	2.150553608	Q14209
Myogenin	4.428352557	2.10436512	P15173
BMX	4.287625586	2.070658249	P51813
Roaz	4.147520675	2.036546261	O08961
Stat 2	4.026073388	2.006507759	P52630
XPD	3.728023836	1.930809114	P18074
DDX1	3.543080696	1.88230728	Q92499
Gelsolin	3.24618897	1.801718338	P06396
Rnase HI	3.034931988	1.742105619	O60930
IP3R-3	2.963121867	1.721372088	Q14571
b-PIX	2.922837517	1.709630813	Q14155
K+ Channel a	2.87984082	1.697009375	Q9BX94
Fnk	2.846153265	1.687054612	Q9H4B4
TRAF2	2.825144021	1.680816475	Q12933
CD28	2.768599463	1.663910894	P10747
NEK2	2.750415852	1.658437775	P51955
FXR2	2.663149681	1.631915954	P51116
Caspase-4 / TX ICH-2	2.620710371	1.618860825	P49662
BM28	2.466187202	1.570409884	P49736
MGMT	2.447587524	1.564476757	P16455
MDM2	2.378792905	1.542333591	Q9UMT8
PKA rIIb	2.290908937	1.513574887	P31323

Table 3 continued

Downregulated			
Ab-Ag	**Average R/R**	**Average INR**	**Accession #**
PARP	0.142816133	0.377910218	P09874
NHE-1	0.171849378	0.414547196	P19634
Dynamin I	0.335897099	0.579566303	Q05193
p63	0.33842649	0.581744351	Q9UBV9
WT1 (Wilm's Tumor Prot	0.383450928	0.619234147	P19544
CDC34	0.384927678	0.620425401	P49427
IL-13	0.392134164	0.626206167	P35225

2.6.2 Analysis of Common Promoter Elements

Materials and Methods:

In order to determine whether common motifs exist in the genes found to be up- and downregulated by Vpr, the promoters of six of these genes were mapped and alignment studies carried out. The sequence of the promoter region of each gene was attained using the web based program TRASER (Transcript Sequence Retrieval, http://genome-www6.stanford.edu/cgi-bin/Traser/traser). TRASER can generate up to 2000 base pairs of lead sequence for a given accession number. Common motifs were investigated using several alignment and motif scanning programs, which yielded lists of small potentially conserved regions. A more logical approach was devised to map potential transcription factor binding sites and determine common sites and patterns between genes. Predicted transcription factor binding sites within each promoter were determined using TESS (Transcription Element Search System, http://www.cbil.upenn.edu/cgi-bin/tess/tess), which is a user-friendly interface for the TransFac database. A putative map was drawn using the sites with the highest predicted value via TESS and showing evolutionary conservation across species and cross referenced with that predicted using the Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

Results and Discussion:

The introduction of high throughput arrays has exponentially increased the amount of data attained from expression studies. New technologies have streamlined the experimental methods, such that analysis of the data generated is now the limiting factor. Several attempts have been made to introduce software packages for microarray analysis, but these programs have

remained limited and cumbersome to the average user. Currently available analyses are aimed at recognizing molecular "footprints" for disease states, such as prediction of severity and metastasis in cancer, and are not appropriate for array data intended to be hypothesis generating. Thus, end users of array data must devise methods of analysis to conjugate their disjointed lists of genes into useful models and hypotheses. In this section, I describe the analysis of array data by promoter prediction.

HIV-1 Vpr is a small, virally synthesized protein. Although it is difficult to measure its "concentration" *in vivo*, we can predict that as a viral protein it is not made in great abundance by the cell. In Section 2.4.1 we reported altered expression of 69 cellular genes at the mRNA level and in Section 2.6.1 we report altered expression of 20 cellular proteins. While it may be possible, it is not probable that Vpr is forming unique complexes to individually modulate the expression of each of these genes. The ability of Vpr to form stable complexes capable of remaining associated for detection through techniques such as immunoprecipitation is evidence against the immensely high dissociation rate which would be necessary for formation of a large number of alternate complexes. Thus, we predict that common motifs exist in the regulatory regions of these genes which are mediating their regulation by Vpr. In order to test this hypothesis, I generated putative promoter maps for six genes found to be regulated by Vpr, either by microarray or antibody array analyses. To look for common motifs governing Vpr-mediated downregulation, I chose to analyze NHE1, HSP40 (DNAJ), and TR3 (Nur77). To look for motifs governing upregulation by Vpr, I chose to analyze TNF alpha, HSF1, and synaptotagmin. These genes were some of the most highly regulated in the previous analyses, making it more probable that if patterns exist they will be discernable in this sample. Sequence retrieval, promoter prediction, and transcription factor binding site determination were carried out using the web based programs cited in Methods. The results of each analysis was a map of putative transcription factor binding sites within a promoter. Literature searches of studied regions of each of these promoters were used to confirm mapping. Due to its further study in Specific Aim 3 (Chapter 4), the putative promoter of TNF alpha is included as the example map below (Figure 5).

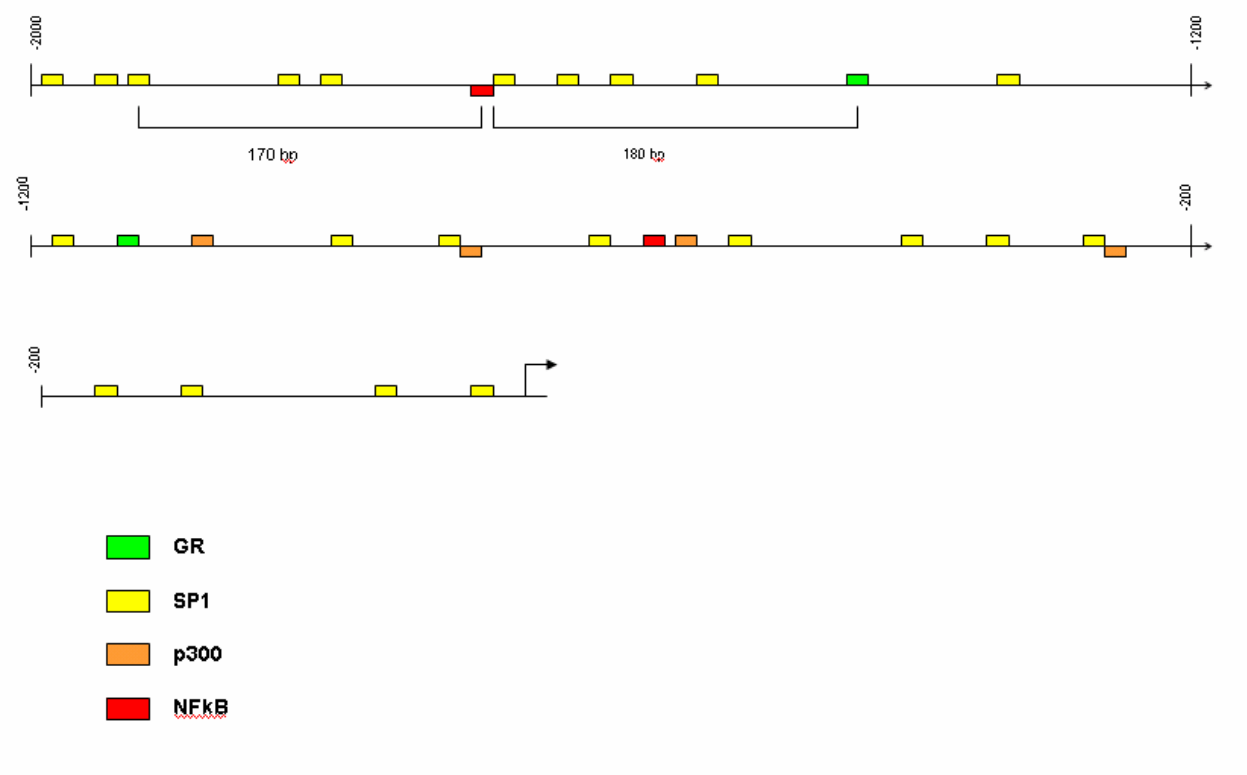


Figure 5: Predicted transcription factor binding sites within the promoter of TNF alpha.
The sequence of the upstream region of the TNF alpha genes was input into the program TESS in order to generate potential transcription factor binding sites. Sites were chosen for inclusion in the above map based on high predicted value score in TESS and/or evolutionary conservation. Emphasis is given to known binding partners of Vpr. The legend indicates the transcription factor for each colored block on the map. GR, glucocorticoid receptor.

Visual inspection of the six maps generated revealed several patterns. Within the upregulated genes, all three contained triplicate Sp1 sites and a GR binding site within 200 base pairs of one another. This pattern is interesting, as it is similar to that present in the HIV-1 LTR. Vpr has been shown to specifically bind to both Sp1 (Wang, 1995, Amini 2004) and GR (Vanitharani, 2001) to affect transcription from both the LTR and cellular genes. Thus, these common motifs present a potential site for Vpr mediated regulation of expression. Vpr has also been shown to affect transcription through NF-kB and p300, but binding sites for these transcription factors were not present in all three sequences queried here. The relevance of the promoter region in determining Vpr-mediated upregulation will be further studied for TNF alpha in Chapter Four. Among the downregulated genes, all three possessed an Oct-1 binding site

within 170 base pairs of the start of transcription. This spacing is also similar to the LTR, which contains an Oct-1 binding site at -165, as well as three additional Oct-1 sites which are further upstream of -165. At present, no studies have linked Vpr directly to Oct-1 mediated repression of transcription. Though, in the context of viral infection, motifs similar to the LTR may be favored by transcriptional complexes in part formed by viral proteins such as Vpr. Of note, the TNF promoter also contains an Oct-1 binding site (Knight, 1999), though farther upstream than the one described as a common motif here. It was not noted on the original predicted map included in Figure 5 due to its low predicted value via TESS, but studies have clearly shown it is important in regulation of TNF alpha. This information indicates the potential downfall of predicted values for transcription factors binding. Though bioinformatics have become more powerful predictors of biological significance, high rates of inconsistency still plague these tools (Qiu, 2003). Thus, we will consider the data attained here as hypothesis generating, providing the basis for future directions in addressing differential transcription factor binding patterns in HIV infected cells. First, though, the remainder of this study will be dedicated to confirming the observed regulation of two of the genes found to be regulated in Specific Aim One, NHE1 and TNF alpha, and determining their potential roles in mediating the biological functions of Vpr.

3.0 CHAPTER 3: HUMAN IMMUNODEFICIENCY VIRUS (HIV-1) VPR INDUCED DOWNREGULATION OF NHE-1 CORRELATES WITH LOSS OF THE ANTI-APOPTOTIC FUNCTIONS OF NHE-1

Portions of this chapter were modified from

Janket ML, DeRicco JS, Borowski L, and Ayyavoo V.

Human Immunodeficiency virus (HIV-1) Vpr induced downregulation of NHE-1 correlates with loss of the anti-apoptotic functions of NHE-1.

Under preparation.

3.1 PREFACE

The original abstract and introduction for the prepared manuscript were not included here in order to maintain readability within the present study. The manuscript includes information from Specific Aim One/Chapter Two, the inclusion of which in this chapter would be redundant. Thus, a revised abstract and brief introduction to the material presented in the Chapter has been inserted.

3.2 ABSTRACT

One of the most highly downregulated proteins attained via array data generated in Chapter Two was the sodium hydrogen exchanger, isoform 1 (NHE1), which showed a 60% decrease in expression in HIV-1 vpr+ infected cells compared to HIV-1 vpr- treated control. Downregulation of NHE1 was specific, as expression of NHE3 (isoform 3) was not altered. The goal of this study was to confirm the downregulation of NHE1 by HIV-1 Vpr, as well as to determine the potential cellular consequences of NHE1 downregulation. Western blot analysis was used to first confirm the results of the protein array. Additionally, real-time RT-PCR analysis in multiple donor PBMCs (n=8) showed that down regulation of NHE1 occurs at the level of transcription ($p<0.005$). Vpr-mediated NHE1 downregulation was reversed by mifepristone, indicating that this is occurring through the glucocorticoid receptor (GR) binding activity of Vpr. NHE1 is involved in maintenance of cellular pH through its hydrogen exchange capacity at the cell surface. The presence of Vpr induced acidification of infected host cells as compared to those infected with vpr- virus, as would be expected due to reduced NHE1 exchange due to Vpr mediated downregulation. NHE1 has been described to play an important scaffolding role in the Akt kinase pro-survival signaling pathway. Here, we have found that

Vpr-mediated downregulation of NHE1 correlated with decreased expression of the phosphorylated form of the pro-survival Akt kinase. The results of this study therefore present one of the potential signaling pathway(s) contributing to the induction of apoptosis which has been described for Vpr.

3.3 INTRODUCTION

HIV-1 Vpr induces cell cycle arrest and apoptosis in infected cells. While it is well accepted that persistent exposure to Vpr induces apoptosis, the mechanism mediating this effect is unknown. As described in Section 1.2.9, studies have suggested caspase activation (Muthumani, 2002), Vpr binding proteins (Miles, 2005), induction of the ATR-mediated DNA damage response (Anderson, 2005), and disturbance of the mitochondrial membrane potential (Roumier, 2002) as potential mechanisms. The aforementioned mechanisms, such as caspase activation and loss of mitochondrial membrane integrity, all represent downstream events in apoptosis. Studies to date have failed to produce the upstream mediators of Vpr-mediated apoptosis. In an effort to determine potential cellular proteins whose expression may be linked to the functions of Vpr, we conducted the array analyses described in Chapter Two. From that analysis, we determined that one of the most highly downregulated cellular proteins was NHE1.

NHE1 is isoform 1 of the sodium hydrogen exchanger family (reviewed in Fliegel, 2005). Sodium hydrogen exchangers function at the cell membrane to exchange intracellular hydrogen ions (H+) generated during cellular metabolic processes for extracellular sodium ions (Na+). In maintaining the balance of these two ions, NHE1 also maintains both the intracellular pH and cell volume at homeostatic levels. Reduced capacity of NHE1 to perform either of these functions has been shown to induce cellular apoptosis. Fluxuations in intracellular pH mediated by NHE1 activity have also been linked to cell cycle control, especially of arrest in the G2 phase (Putney, 2003). A recent paper by Wu et. al. (2004) has discovered a role of NHE1 in maintaining cell survival which is separate from its Na+/H+ exchange capacity. Thus, given the dual role of NHE1 as an anti-apoptotic protein, a reduction of NHE1, such as that noted by antibody array, might be expected to lead to induction of host cell apoptosis. The goal of the work presented in this aim is to confirm the downregulation of NHE1 noted in Chapter Two

using alternate techniques, as well as to determine if NHE1 downregulation is associated with loss of anti-apoptotic properties of NHE1. The effect of NHE1 on both apoptosis and cell cycle arrest, both well described functions of Vpr, as well as the existence in its promoter region of both GR and Sp1 sites, known transcription factor binding partners of Vpr, make NHE1 a potential player in Vpr biological functions.

3.4 MATERIALS AND METHODS

Cells and Transfection

Blood from HIV-1-negative, healthy donors was used to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (Pharmacia) gradient centrifugation. Purified PBMCs were resuspended in RPMI 1640 supplemented with 10% FCS, stimulated with phytohemoagglutinin (PHA) (5μg/ml) for 3 days, and cultured in IL-2 (5U/ml) containing medium. HEK293 and HEK293T (American Type Culture Collection, ATCC) cells were maintained in DMEM supplemented with 10% FCS, 1X L-glutamine, and 1X Penicillin/Streptomycin. CEMx174 (NIH AIDS Reagents and Reference Program, NIH-ARP) cells were maintained in RPMI-1640 supplemented with the same supplements.

Where indicated, HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen), as per manufacturer's instructions. Briefly, HEK293 cells were plated on 100mm plates and grown to 70-80% confluence. Transfection mixture was made containing Opti-MEM (Gibco), Lipofectamine 2000 (10ul/100mm plate) and vector (pcDNA3.1, 1.0μg), *vpr* expression plasmid (pcDNA3.1vpr, 1.0μg) or eGFP plasmid control and added dropwise to plates. Following 12-14 hours post-incubation, transfection medium was removed and replaced with complete medium (supplemented as above). Transfection efficiency was determined by observation of adjacently eGFP transfected plates under an inverted fluorescent microscope and only experiments with ≥40% transfection efficiency were considered for analysis. Cells were collected 48 hours post transfection for RNA extraction and real-time RT-PCR analysis.

Plasmids and Virus Infection

HIV-1 proviral DNAs 89.6wt (wild type with an intact *vpr* gene), and 89.6*vpr*- (containing a frameshift mutation in the Vpr coding sequence to eliminate its expression) were constructed as described (Baillet, 1994). PHA-stimulated normal human PBMCs (10 x10^7) were infected with viruses containing 100 ng p24 antigen equivalent of 89.6wt or 89.6*vpr*- virus stocks as described (Baillet, 1994). For single round infection, HIV-1 pNL43 E- *vpr*+ and pNL43 E- vpr-- constructs were pseudotyped with vesicular stomatitis virus (VSV-G) envelope, denoted as HIV-1 vpr+ and HIV-1 vpr- respectively. HEK-293T cells (2x10^6 per plate) were co-transfected with 15.0µg of HIV-1 proviral construct (pNL43 *env- vpr*+ or pNL43 *env- vpr*-) and 5.0µg VSV-G-Envelope (VSV-G-Env) expression plasmid by the calcium phosphate precipitation method. Non-enveloped virus was prepared in the same manner, with the omission of VSV-G. Cell free supernatant was collect 72-120 hours post transfection and assayed for virus production via p24 ELISA. Number of infectious particles was determined by titration on the TZM cell line as described (NIH-ARP). Cells (as indicated per experiment) were infected at an MOI of 0.5 to 2.0, as indicated in figure legends. Percentage of infected cells was determined by intracellular p24 staining (Beckman Coulter Clone KI57) and analysis by flow cytometry.

Immunoblot Analyses

Normal human PBMCs were cultured and infected as described above. Whole cell lysates were prepared in Triton X-100 lysis buffer containing; 1M Tris pH 7.5, 5M NaCl, 10% Triton, 100mM Na3VO4, 1M NaF, 50mM PMSF, Aprotanin, Pepstatin, Leupeptin, and Chymostatin. Protein concentration was assessed by Bio-Rad Protein Assay (Bio-Rad) and 10-50µg equivalent of protein was electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel (SDS-PAGE), and proteins were transferred to PVDF membrane (Millipore). Immunoblot with the following primary antibodies was carried out at either room temperature for one hour or overnight at 4°C as per manufacturer's suggested protocol; mouse monoclonal anti-NHE1 (1:250, BD Transduction Laboratories), rabbit polyclonal anti-Vpr (1:500, kind gift of John Kappes, University of Alabama), anti-Gag (1:500, NIH-ARP #4121), anti-alpha tubulin (1:500, NeoMarkers), anti-Akt-P (serine 473, 1:250, Cell Signaling), anti-Akt (1:250, Cell Signaling). After washing three times in Tris-buffered saline- Tween-20 (TBS-T), membranes were incubated one hour at room temperature in appropriate goat anti-mouse or goat anti-rabbit HRP-

conjugated secondary antibodies (1:5000, Caltag). Following five washes in TBS-T, antibody bound proteins were detected by autoradiography using Pierce SuperSignal West Pico system.

Quantitative RT-PCR using Taqman.

To confirm the expression ratios seen with antibody array, the mRNA levels were quantified by real-time RT-PCR. Cells (PBMCs or HEK293) were treated as described above, collected by centrifugation, and washed once with phosphate buffered saline. RNA was extracted using Qiagen RNeasy mini kit as per manufacturer's instructions, with optional on-column DNase digestion (RNase free DNase kit, Qiagen). RNA integrity and concentration were assessed by agarose gel electrophoresis and spectrophotmetry. RNA (1µg per triplicate reaction) was reverse transcribed to cDNA using the Taqman Gold Reverse Transcription kit (applied Biosystems). Real-time RT-PCR was carried out using 2X Taqman Universal Master Mix without UNG and NHE1 specific primer and probe mix (#Hs01011912_g1, 20X), both Applied Biosystems. Data was collected on an ABI PRISM 7000 and analyzed via Sequence Detector v1.1 software. All values were normalized to the endogenous control RPLPO to control for variation in RNA integrity, concentration, and pipetting and human errors. Assays were repeated in triplicate and average threshold cycle (CT) values were used to determine relative concentration differences based on the ΔΔCT method of relative quantitation described in the manufacturer's protocol, User Bulletin #2 (ABI, 1997). Statistical significance of difference between mean of experiments conducted multiple times was computed using Repeated Measures ANOVA to account for the intra-experiment (assay run in triplicate) and inter-experiment (four separate experiments) errors. This method considers both variation between experiments and repeated measurements within each experiment, thus allowing for prediction of significance with higher confidence.

Flow Cytometry.

Confirmation of infections: All infections carried out in primary human PBMCs were assessed by flow cytometry. Cells were collected by centrifugation, washed twice in phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde for one hour at 4°C. Following fixation, cells were permeabilized for intracellular staining through incubation in buffer containing 0.1% saponin (Sigma) in PBS at room temperature for >10 minutes. Cells were again

collected by centrifugation, supernatant was removed and cells were resuspended in the residual permeabilization buffer. Intracellular staining for p24 antigen was carried out by addition of 2.5µl of anti-p24 FITC conjugated (clone KI57, Beckman Coulter) for 1-2 hours at 4°C. Cells were washed twice in permeabilization buffer to remove excess antibody, resuspended in PBS containing 5% FBS and analyzed on a Beckman Coulter XL-MCL using System II software. Post-data collection analysis was done using CellQuest software.

Intracellular pH Staining and Flow Cytometry

The pH sensitive dye 5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetate was used to assess intracellular pH. SNARF-1 is excited at one wavelength and emits at two wavelengths, with the ratio of fluorescent intensity between the two wavelengths varying with pH. The AM ester is impermeant to cells once it has broken down by intracellular esterases, allowing for less leakage of the dye while performing flow cytometry. Stock solution of SNARF-1 was made at 10mM in cell culture grade DMSO, and diluted to 10µM in PBS for staining. Cells to be stained (1x10^6) were washed twice in PBS and resuspended in SNARF-1. Dye loading was carried out at room temperature for 30 minutes in the dark. Cells were washed twice, resuspended in 1ml PBS and immediately analyzed on a Beckman-Coulter XL-MCL using SystemII. Dye was excited at 488nm, fluorescence was measured using 575BP and 645DLP filters.

The ratio of the mean fluorescence at 645BP/575BP is reported, followed by the change in the ratio between treatments and the change in intracellular pH this represents, as calculated from the calibration curves. In order to generate calibration curves for each cell type used, uninfected PBMCs, CEMx174, or Jurkat cells were stained as described above. Following staining, cells were incubated in high K+ buffers of known pH (range 6.0-9.0) as per the method of Bond and Varley (2005). Nigericin (2ug/ml, Sigma) was added to the buffers in order to equilibrate the intracellular pH to the extracellular buffer pH. Nigericin is an ionophore which disrupts membrane potential by allowing the free passage of ions across the plasma membrane. In this experiment, it allows free passage of H+ and K+, equilibrating the intracellular environment to that of the extracellular buffers of known pH. Flow cytometry was performed as described above. A graph of input pH versus fluorescence ratio was generated and a third order polynomial best fit curve was generated as referenced in Bond and Varley (2005). The equation

of the best fit line was used to compute changes in pHi from change in mean fluorescent intensity. Values reported are the mean of four experiments.

3.5 RESULTS

The Na/H exchanger, isoform 1 (NHE1) is specifically downregulated by Vpr

Gene expression analyses described above indicated the potential role of Vpr in mediating changes in cellular apoptotic pathway signaling. Such findings are in agreement with previously published studies which describe the ability of Vpr to induce cell cycle arrest and apoptosis (reviewed in Section 1.2.9). Based upon these two known downstream functions of Vpr, we chose to focus on the effect of Vpr on the Na+/H+ exchanger. Isoform 1 of the sodium hydrogen exchanger was found to be downregulated by a ratio of 0.41 by antibody array analysis. Results from the antibody array suggested that downregulation of NHE1 was specific, as NHE isoform 3 was not affected by Vpr (ratio 1.01). In order to confirm the specific downregulation of NHE1 by Vpr, we employed western blot analysis using an NHE1-specific antibody. Peripheral blood monouclear cells (PBMCs) isolated from healthy donors were stimulated for three days followed by infection with HIV *vpr*+ or HIV *vpr*- (see Methods). Seventy-two hours post infection (peak infection in the single cycle system as assessed previously in our laboratory), cells were assessed for percent infection by intracellular staining for p24 antigen and flow cytometry. Cells showed a similar percentage of infection between HIV *vpr*- and HIV *vpr*+ infected cells. In addition, cell death, as measured by trypan blue staining, was low and relatively equivalent at 5.2 and 3.9% in HIV *vpr*+ and HIV *vpr*- infected cells respectively. Percentage infection and cell death are therefore similar between samples and are regarded as negligible confounding variables. Cells were collected, lysed, and subjected to

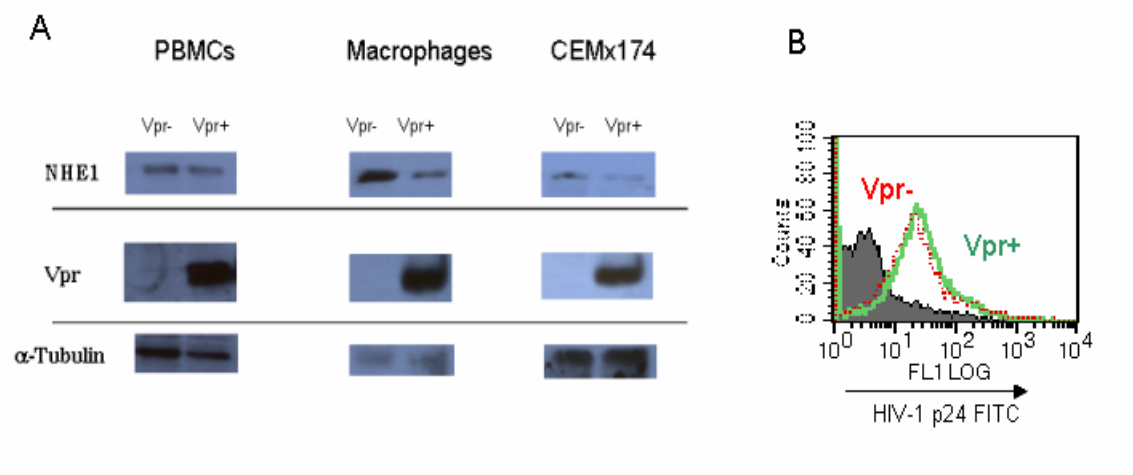


Figure 6: Confirmation of NHE1 downregulation by western blot.

Total PBMCs and macrophages from healthy donors or the cell line CEM x174 were infected with HIV vpr+ or HIV vpr- virus. Seventy –two hours post-infection, cells were collected, assessed for infection, lysed, and subjected to immunoblot with the indicated antibodies. (A) Immunoblot analysis of NHE1 in HIVvpr+ or HIVvpr- virus infected cells. Anti-p24 and anti-tubulin are shown as controls for viral infection and protein loading, respectively. Results are representative of four experiments. (B) Flow cytometry analysis to determine the infectivity of HIVvpr+ and HIVvpr- virus. PBMCs infected as above were fixed, permeabilized, and stained for intracellular p24 antigen expression using FITC conjugated anti-p24 antibody. Flow cytometry was carried out on a Beckman Coulter XL-MCL and analysis completed using CellQuest software. Overlay shows HIVvpr+ in green and HIV vpr- in red. Shadowed histogram indicates the IgG-FITC control antibody

SDS-PAGE followed by western blot with the specific antibodies noted in Figure 6A. Results indicate that NHE1 is specifically downregulated by Vpr in the context of infection. To further confirm this finding, multiple normal donors PBMCs were infected with 0.5-1.0 MOI of HIV-1 *vpr+* or HIV-1 *vpr-* virus and assessed for NHE expression. Results indicate that NHE expression was downregulated in the presence of Vpr in a dose dependent manner without altering the total cellular protein level (alpha-Tubulin control) suggesting that Vpr-mediated down regulation of NHE is specific. Similar results were observed in multiple donors. Given the ability of HIV to infect different cell types within the immune system, we next determined whether Vpr-mediated NHE1 downregulation is cell type specific. Similar immunoblots were conducted in macrophages and the cell line CEMx174. NHE1 was found to be downregulated in each of these cells types, in multiple donors (for the macrophages), indicating that Vpr-mediated NHE1 downregulation is not cell type specific.

Vpr mediated NHE1 downregulation is at the level of transcription

Previous studies in our laboratory have shown the ability of HIV-1 Vpr to influence expression of host genes at the level of transcription. To asses whether the downregulation of NHE1 is induced at the transcriptional level, we employed real-time RT-PCR. Patient PBMCs infected as described above with HIV vpr+ or HIV vpr- viruses were collected and total RNA

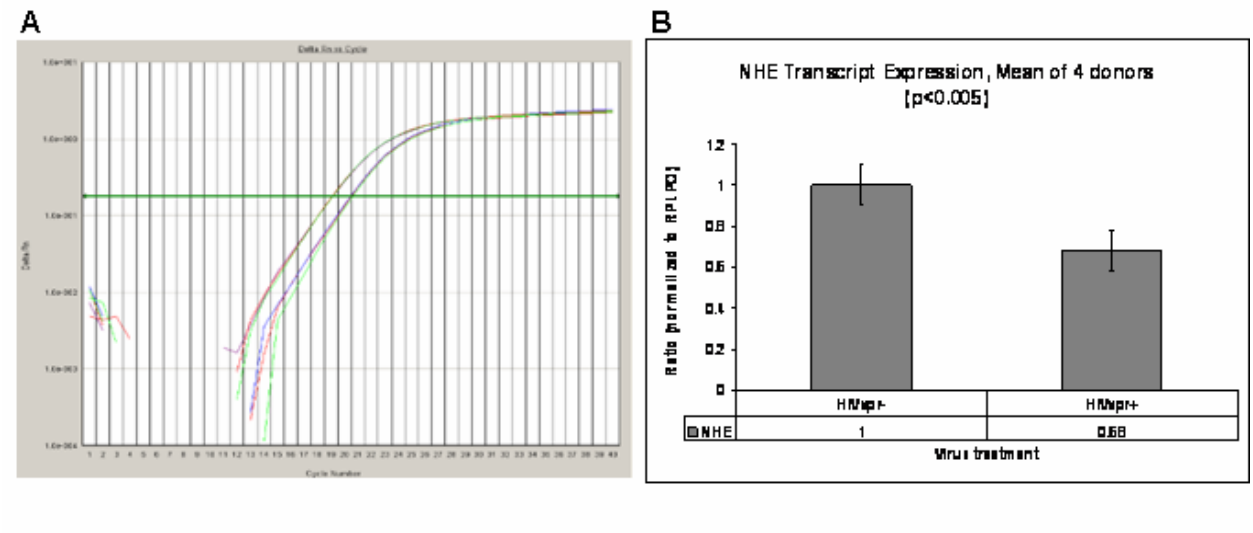


Figure 7: Real time RT-PCR analysis of NHE1 expression.

Healthy donor PBMCs infected with HIVvpr+ or HIVvpr- were collected 72 hours post infection and mRNA extracted using the Qiagen RNeasy mini kit. One ug per triplicate reaction was reverse transcribed to cDNA and real time PCR was carried out using primers and probe specific for NHE1 or RPLPO. (A) Raw data displayed as an amplification plot. Delta Rn indicates the fluorescence intensity from the probe corrected for background and cycle is the PCR cycle. The horizontal line represents the threshold level of expression considered for each gene. (B) Mean of four experiments in PBMCs. Ratios were generated using the ΔΔCT method of relative quantitaion. Values were normalized to the endogenous control RPLPO. In each experiment, the results attained in HIVvpr- infected cells were considered as ratio of 1.0.

was extracted and subjected to real time RT-PCR using NHE1 specific primers and Taqman probes. The levels of the housekeeping gene RPLPO (human ribosomal large protein) were used as an internal control for RNA integrity and human error, as RPLPO has been shown to maintain

consistent levels of RNA in activated lymphocytes (Lossos, 2003). All values were normalized to RPLPO and calibrated to the HIV vpr- sample using the ΔΔCT method of relative gene quantitation (ABI User Bulletin 2). All samples were tested in triplicate and a representative amplification plot is shown in order to demonstrate the high level of internal consistency and low standard error in these experiments, yielding the high statistical significance reported below (Figure 7A). In all donors tested, NHE1 was found to be downregulated by a ratio of approximately 0.5, similar to that seen in the antibody array. The average and standard deviation results from four donors are shown, indicating that NHE1 was downregulated by Vpr in a statistically significant manner ($P<0.005$). Taken together, the results of the immunoblot and real time RT-PCR indicate that NHE1 is downregulated by Vpr at the transcriptional level.

Effect of NHE1 regulation by Vpr in the absence of other viral proteins and/or infection

The results above indicate that Vpr induces downregulation of NHE1 in the infected cell. In the infected host, Vpr is present in multiple forms; virion associated, de novo expressed, and free Vpr protein. Vpr released from degraded virus particles or from the infected cell could be easily taken up by the uninfected bystander cells via transduction. In order to assess the potential effect of Vpr on NHE1 in these diverse contexts, we tested the effect of Vpr in the absence of other viral proteins. HEK293 cells were transfected with control vector (pcDNA3.1) or Vpr-expression plasmid and the level of NHE1 mRNA was assessed by real-time RT-PCR as described in Methods. Considering the NHE1 level in vector transfected cells as 1, the Vpr transfected cells showed a ratio of 1.08 indicating that Vpr in the absence of viral infection did not induce NHE1 downregulation in a manner similar to infection (Figure 7A). These results support our theory, presented in Chapter 2, that Vpr may have different functions in infected versus uninfected cells. As such, we further confirmed this finding with an alternate means of transfection. NHE-1 is localized at the cell membrane and its activity may be in part controlled by its rate of recycling similar to other NHE isoforms. It is therefore possible that disruption of the cell membrane via fusion of the lipid vesicle utilized in transfection may have disrupted the recycling of this protein, thus altering the expression. In order to confirm that transfection did not downregulate NHE-1, experiments were repeated using calcium phosphate, a non-fusion mediated transfection method. Results were similar to those attained via lipid mediated

transfection (Figure 7B), with Vpr transfected cells expressing NHE-1 at a ratio of 1.16 compared to vector transfected cells. These results confirm that de novo synthesized Vpr, in the absence of virus infection, is unable to induce NHE-1 downregulation, given the inability of Vpr to mediate NHE1 downregulation in the absence of viral infection.

Next to confirm whether the infection process is required to observe the Vpr-mediated NHE1 regulation, we infected the HEK293 cells with the VSV-envelope pseudotyped HIV-1 vpr+ or HIV-1 vpr- virus. Seventy-two hrs post infection RNA was isolated and analyzed for NHE1 level (Figure 7C). Results indicate that NHE1 was inhibited by 50% in HEK293 cells infected with HIV-1 vpr+ virus (0.532) compared to HIV-1 vpr- virus infected cells (1.0). These results further confirm that Vpr alone in the absence of virus infection did not induce any changes in NHE1 indicating that some component of viral binding, fusion, or infection is necessary for Vpr-mediated NHE1 downregulation.

In light of these interesting results, we further explored whether the addition of cellular stress could restore the ability of Vpr to induce NHE-1 downregulation. The host cell antiviral response to double stranded RNA viruses, such as HIV, includes the induction of cellular "stress" pathways, which attempt to restore normal functioning to the cell (Haines, 1991). Upon HIV-1 infection, studies have found induction of the heat shock response (Agnew 2003, Kocsis, 2003), cell cycle arrest (Howett, 1995, Kolesnitchenko, 1991), and DNA repair pathways (Daniel, 2003). The induction of all three of these pathways is thought to be mediated in part by Vpr, but may require the presence of other viral proteins. Stress responses have been shown to be important in the control of NHE-1, most notably cell cycle (Besson, 1998) and heat shock responses. Thus, we sought to determine whether providing these stress responses in trans, as a mimic of viral infection, may restore the ability of Vpr to induce NHE-1 downregulation. The use of well studied stress induction pathways to restore function may also provide a specific signaling pathway to be explored in determining the mechanism of Vpr-mediated NHE-1 downregulation. In order to mimic the three stress conditions cited above, we chose to subject HEK293 cells, at 24 hours post Vpr transfection, to heat shock, serum starvation, or ultra-violet (UV) light exposure. Heat shock at 42°C for ≥30 minutes has been used successfully in our laboratory to induce the heat shock response, as determined by increased expression of inducible heat shock protein 70 (HSP70) transcript level at two hours post heat shock. Serum starvation

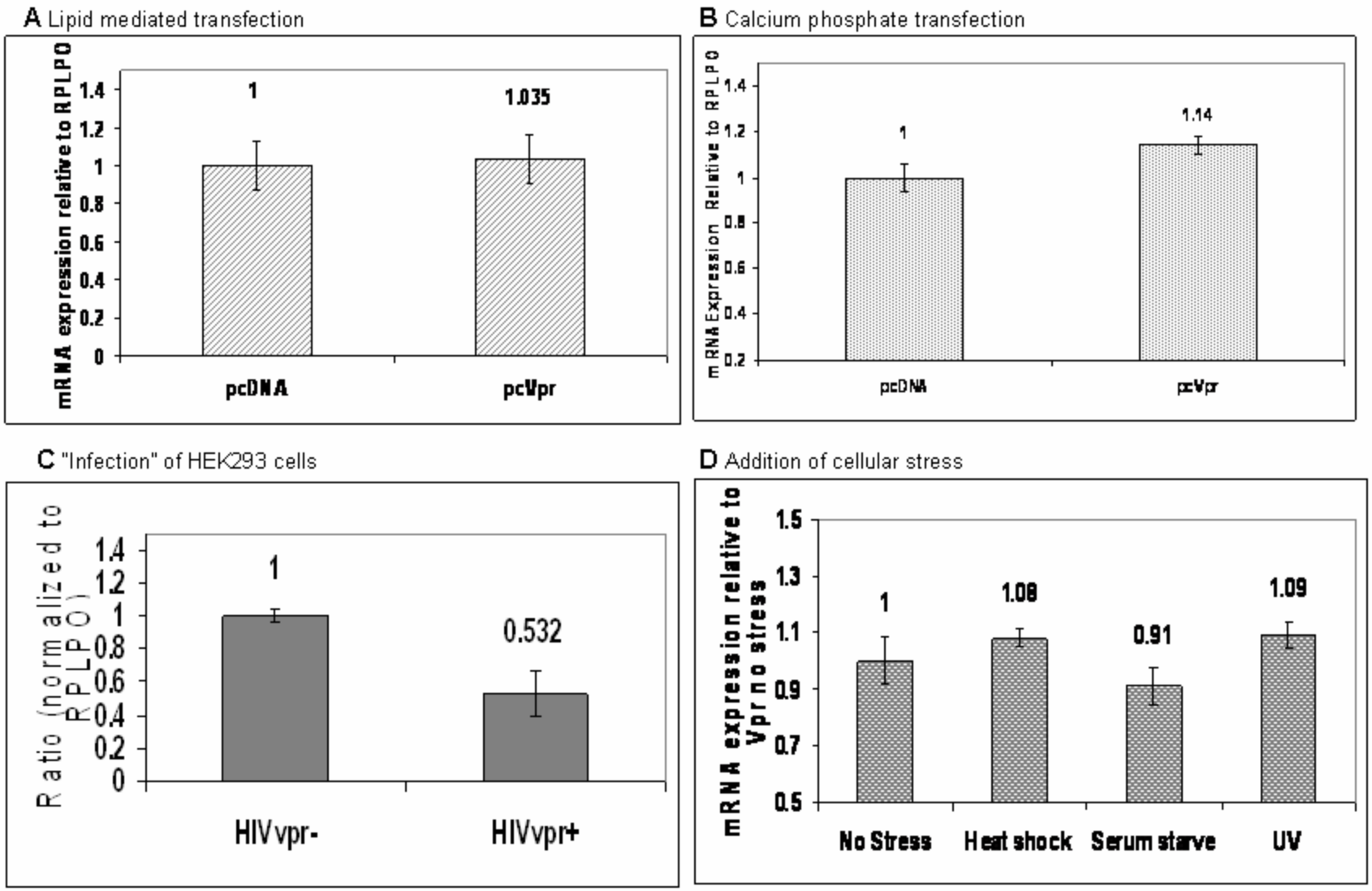

A Lipid mediated transfection
mRNA expression relative to RPLP0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
1.035
pcDNA
pcVpr
B Calcium phosphate transfection
mRNA Expression Relative to RPLP0
1.4
1.2
1
0.8
0.6
0.4
0.2
1
1.14
pcDNA
pcVpr
C "Infection" of HEK293 cells
Ratio (normalized to RPLP0)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
0.532
HIVvpr-
HIVvpr+
D Addition of cellular stress
mRNA expression relative to Vpr no stress
1.5
1.3
1.1
0.9
0.7
0.5
1
1.08
0.91
1.09
No Stress
Heat shock
Serum starve
UV

Figure 8: Effect of de novo Vpr on NHE1 in the absence of HIV viral infection.
HEK293 cells were transfected with either pcDNA3.1 (vector) or pcDNA3.1vpr. Forty-eight hours post transfection cells were collected and assessed for Vpr expression and NHE1 regulation. Total RNA was extracted from cells transfected with control or vpr-containing plasmid by lipid micelle (A and D, Lipofectamine 2000) or calcium phosphate methods (B) or infected with HIVvpr+ or HIVvpr- pseudotyped with VSV-G envelope (C). Real time RT-PCR was carried out using NHE1 or RPLPO specific primers and probes and analysis conducted using the relative quantitation method. Means of four (Lipofectamine 2000), three (calcium phosphate), and four (cellular stress) experiments are shown.

was utilized to mimic cell cycle arrest and UV light exposure to induce the DNA damage repair pathway. Results indicate that none of the three stresses tested were able to restore the ability of Vpr to induce NHE-1 downregulation in the absence of HIV-1 infection (Figure 7D). Since virus replication is a more prolonged process than transient stress induction, the possibility existed that NHE-1 regulation in the absence of virus replication may be transient and thus released following the reintroduction of cellular homeostasis. To ensure that we did not miss an early, transient change in NHE-1 expression, NHE-1 mRNA levels were also assessed at two hours post stress induction and no change was seen from that attained at 24 hours post stress induction. Thus, in the absence of an effect of a general cellular response pathway, further study will be necessary to determine the specific HIV proteins and/or processes necessary to compliment Vpr and induce NHE-1 downregulation

Vpr-mediated NHE downregulation results in change in intracellular pH

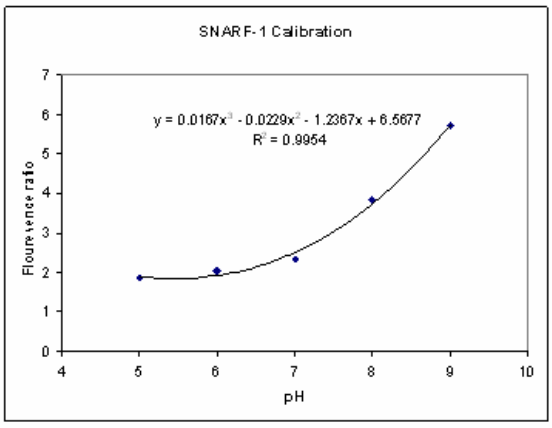
NHE1 functions as a sodium hydrogen exchanger at the cell surface, exchanging intracellular H+ ions generated during metabolism with extracellular NA+ ions. NHE1 thus plays a vital role in maintaining intracellular pH, as well as in maintenance of cell volume (reviewed in Putney, 2002). Loss of intracellular pH within the tight range considered physiologically acceptable has been linked to apoptosis (Schneider, 2004). To assess whether Vpr-mediated NHE1 downregulation affects intracellular pH, we performed staining with the pH sensitive dye SNARF-1 (Invitrogen). PBMCs, CEMx174, or Jurkat cells were infected as described above. Seventy two hours post infection, cells were collected and stained with

SNARF-1 as described in Methods. Among the reported concentrations of SNARF-1 used for staining (1μM-20μM, refs), we found 10μM gave the cleanest histogram, with concentrations above this causing greater cell death with no gain in signal intensity. Following the Method of Bond and Varley (2005), we performed calibration by the nigericin method of cellular calibration to generate a standard curve. The resulting values fit well to a third order polynomial equation (Figure 8A, R^2=0.9954) as predicted in published reports (Bond, 2005, Weider, 1993). Average change in intracellular pH was computed from the resulting curves. Results show that the infection, both with HIVvpr+ and HIVvpr- caused some acidification of the cells (Figure 8B). While acidification was mild in HIVvpr- infected cells, with a decrease in pHi -0.1-0.2, the presence of Vpr correlates with a decreased intracellular pH of -0.76 from untreated cells. The tight pH range within which the cell operates makes even small changes in intracellular pH intolerable for normal cellular functioning. Prolonged exposure to Vpr has been shown by ours and other groups to induce apoptosis. Thus, it was important to decipher in this experiment between change in intracellular pH prior to and corresponding with early apoptosis with loss of pH associated with post-apoptotic/necrotic cell death. Cells were stringently gated on live cells via forward and side scatter profile and thus, change in intracellular pH represents that induced by Vpr and not changes expected post-cell death.

Vpr-mediated NHE1 downregulation results in a decreased level of phosphorylation of the pro-survival kinase Akt

Previous publications utilizing mutational studies have shown that in addition to its ion transport domain, the domain of NHE1 responsible for interaction with the ezrin/radixin/moesin complex (ERM) is necessary for cell survival (reviewed in Baumgartner, 2004). The ERM is a family of member microfilament linkers. Recent work by Wu, et. al. (2004) showed that NHE1 at the cell membrane performs a scaffolding function, recruiting ERM to the cell membrane and facilitating interaction of ERM with the pro-survival kinase, Akt. This interaction resulted in increased phosphorylation and activation of Akt, leading to cell survival. Loss of NHE1 at the cell surface led to decreased Akt phosphorylation and cell death. In this study, we were interested

A



B

pHi infected versus uninfected control (NT)				
	Ratio	pHi predicted		
NT	2.559	7.5799		
	HIVvpr-		Δratio (-NT)	ΔpHi
MOI 0.5	2.452	7.3348	-0.107	-0.2451
MOI 1.5	2.482	7.4082	-0.077	-0.1717
	HIVvpr+		Δratio (-NT)	ΔpHi
MOI 0.5	2.407	7.2176	-0.152	-0.3623
MOI 1.5	2.274	6.8193	-0.285	-0.7606

C

Effect of Vpr: pHi HIVvpr+ versus HIVvpr-				
	Ratio	Ratio		
	HIVvpr-	HIVvpr+	Δratio (vpr+-vpr-)	ΔpHi
MOI 0.5	2.452	2.407	-0.045	-0.1172
MOI 1.5	2.482	2.274	-0.208	-0.5889

Figure 9: Intracellular pH in cells infected with HIVvpr+ or HIVvpr-.

PBMCS were infected with HIVvpr+ or HIVvpr- as described and 72 hours post infection cells were collected by centrifugation and washed twice in PBS. Cells were loaded with 10uM SNARF-1 in PBS at room temperature for 30 minutes in the dark. Flow cytometry was carried out on a Beckman Coulter XL-MCL. Live cell gate was set using uninfected PBMCs from the same donor. A) Calibration curve for SNARF-1 staining, third order polynomial is as predicted by Bond and Varley (2004). B) Summary of the mean change in fluorescence intensity ratio and intracellular pH in HIVvpr+ versus HIVvpr- infected cells (four experiments) versus uninfected cells (B) and corrected using HIVvpr- as background to determine the effect of Vpr alone (C). SNARF-1 was excited at 488nm and fluorescent intensity assessed at 575 and 645nm.

in whether the decrease in cellular NHE1 mediated by Vpr affects the level of phosphorylation of the pro-survival kinase, Akt. Normal donor PBMCs were infected and maintained as described in Methods. Seventy-two hours post transfection, cells were collected, and subjected to cell lysis and immunoblot. Lysates used in these experiments were the same as those used in Figure 6, showing decreased NHE1 levels. Immunoblot was carried out using antibodies specific for the phosphorylated form of Akt, as well as unphosphorylated/total Akt. Results indicate that the presence of Vpr correlates with a decreased level of cellular phospho-Akt (Figure 9). Phospho-Akt appeared to decrease in a dose dependent manner with increasing MOI of HIV *vpr+*, while

no change was observed with the highest dose of HIV*vpr*- (MOI of 1.0). Results were repeated in four donors. To further investigate the cell type specificity of this effect, normal donor macrophages and the cell line CEMx174 were both infected and subjected to similar Immunoblot analyses using anti-Akt antibody. Phospho-Akt is decreased in both macrophages and CEMx174, indicating that loss of phospho-Akt in the presence of Vpr is not cell-type specific. This effect was seen in all three macrophage donors tested.

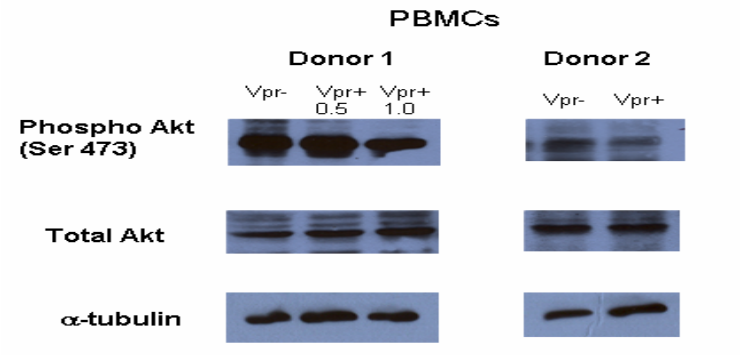


Figure 10: Phospho-Akt in HIV vpr+ and HIVvpr- infected PBMCs.
Cell lysates generated for Figure 6 to assess the level of NHE1 in HIVvpr+ or HIVvpr- infected cells were used in immunoblot for phospho-Akt and total Akt. Equivalent amount of protein (10µg) was separated by SDS-PAGE, transferred to PVDF, and subjected to immunoblot with anti-phospho-Akt (ser 473) or anti-Akt. Alpha tubulin was used as a control for equivalent protein loading. Results are representative of four experiments.

NHE downregulation by Vpr might be in part through GR and could be restored by GR antagonist mifepristone

In the context of normal cells, NHE1 transcription has been shown to be regulated by the glucocorticoid receptor. Specifically, the binding of glucocorticoids to this receptor has been shown to cause a decrease in NHE1 at the mRNA level. (Muto, 2000) Previous work by our group and others have described the ability of Vpr to bind and mediate the transcriptional effects

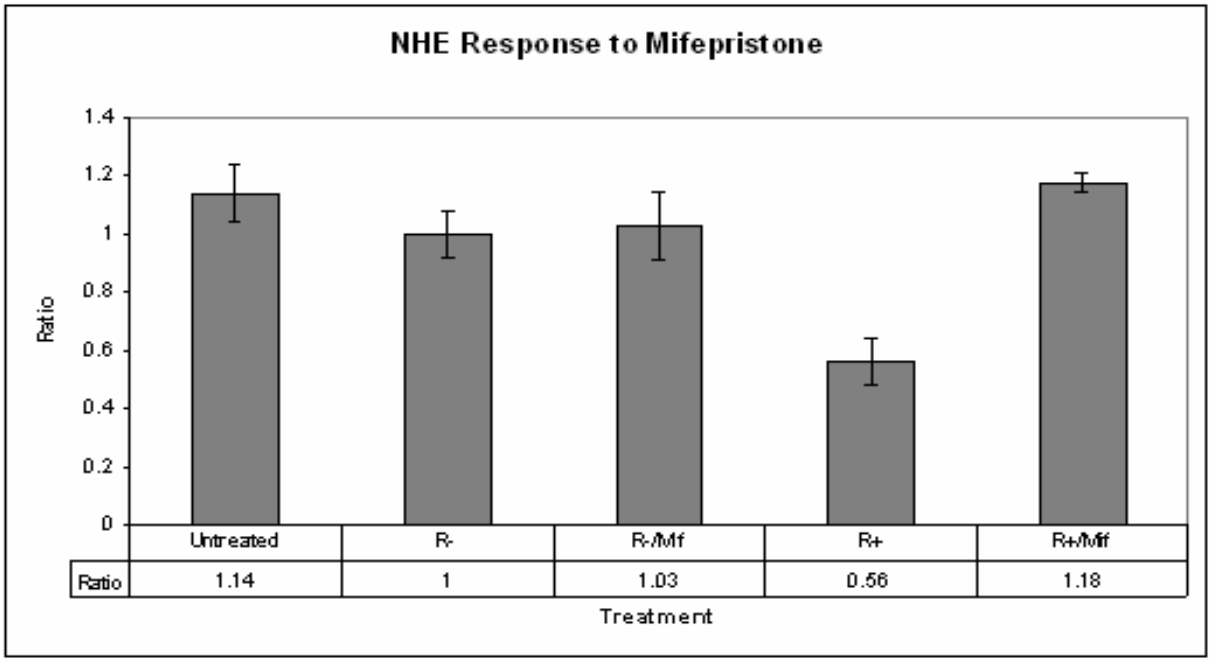


Figure 11: Effect of Mifepristone on NHE1 regulation.

PBMCs were infected with HIVvpr+ or HIVvpr- as described in Methods. Six hours post infection, mifepristone was added to the cells at the doses indicated above. Seventy-two hours post infection, cells were collected and assessed for NHE mRNA expression via real-time RT-PCR. Figure represents the mean of three experiments, error bars represent standard deviation between experiments.

of the glucocorticoid receptor. In order to assess whether this previously described function of Vpr plays a role in downregulation of NHE1, cells infected with HIV-1 *vpr+* and HIV-1 *vpr-* were treated with glucocorticoid receptor antagonist mifepristone and analyzed for NHE1 level (Figure 11). Results indicate that mifepristone reversed Vpr-mediated NHE1 downregulation in a dose dependent manner suggesting that the downregulation of NHE1 may, in part, be affected through the binding of Vpr to the glucocorticoid receptor, a function which has previously been described for this protein.

3.6 DISCUSSION

We chose NHE1 for further study in this chapter based on its previously described role in control of cell cycle (Putney, 2003) and cellular apoptosis (Rich, 2000), two functions which have been well-described for Vpr. NHE1 controls the exchange of intracellular hydrogen ions (H+) generated by metabolic processes with extracellular sodium ions (Na+), thereby maintaining intracellular pH and cell volume within physiologic limits. Such maintenance has been linked to cell survival and cell cycle control. Regulation of NHE1 by Vpr would provide an upstream mediator of these two processes, potentially playing a role in cell cycle-dependent apoptosis induced by Vpr. Additionally, NHE1 has been shown to have a role in anti-apoptotic signaling completely separable from its ion channel functions, through scaffolding cellular proteins at the plasma membrane (Wu, 2004, and reviewed in Baumgartner, 2004). Thus, downregulation of NHE1 may play a role in apoptosis induction at several levels. Results attained in this study indicate that Vpr-mediated downregulation of NHE1 is specific, as no regulation of closely related NHE3 was seen on the antibody array (Results of Chapter 2). In addition, NHE1 downregulation was found to occur at the level of transcription. These results are not surprising, given our previous studies linking Vpr to changes in host cellular gene transcription. What was interesting in these findings was that Vpr, in the absence of viral proteins, was not able to induce NHE1 downregulation. We postulated that some aspect of HIV infection, either entry, replication, or the cellular stress associated with viral infection was necessary in conjunction with Vpr to induce NHE1 downregulation. Broad cellular stressors, including heat shock, serum starvation, and ultraviolet light exposure, combined with exposure to Vpr, did not restore Vpr-mediated NHE1 downregulation. These results indicate that a specific aspect of HIV infection is necessary for Vpr-mediated downregulation, thus we undertook several experiments utilizing different forms of Vpr in order to ascertain whether the necessary steps lie in replication or binding/entry.

Glucocorticoids also stimulate the ion channel activity of NHE1 at short time intervals (3 hours), but eventually induce downregulation of NHE1 at the transcriptional level (Muto, 2004). Thus, because Vpr, does not perfectly mimic the effects of glucocorticoids, perhaps initial stimulation of NHE1 by gp120 binding to the cell surface is necessary prior to downstream

regulation of this gene's transcription by Vpr. Induced stimulation of NHE1 may induce as yet unknown auto regulatory signaling mechanism(s) contributing to its transcriptional regulation.

Results attained in this study suggest that Vpr regulation of NHE1 may present one of the potential mechanism(s) underlying Vpr-mediated apoptosis. In particular, it appears that NHE1 downregulation may be mediated through the action of Vpr on the glucocorticoid receptor. Further study will be necessary to fully delineate the mechanism by which Vpr mediates NHE1 downregulation. Mifepristone has recently been introduced into early phase clinical trials as a novel anti-retroviral agent. Thus, the role of mifepristone in blocking Vpr mediated downregulation may be especially pertinent in understanding the potential effects of this therapeutic agent.

3.7 ACKNOWLEDGEMENT

TZM-bl cells were obtained from Dr. John Kappes through AIDS-ARP, NIH. We thank Dr. John Kappes (University of Alabama at Birmingham) for his gift of anti-Vpr polyclonal antibody used here and Deborah Hollings at Pitt Gene array facility (University of Pittsburgh) for support on Ab array scanning and analysis. This work was supported by grant AI50463 from NIAID, NIH to V.A.

4.0 CHAPTER 4: REGULATION OF TNF ALPHA BY HIV-1 VPR IN INFECTED MACROPHAGES AND ITS CONSEQUENCES IN BYSTANDER CELL SURVIVAL

4.1 INTRODUCTION

In this chapter, we will examine the effect of Vpr on the cytokine tumor necrosis alpha (referred to as TNF alpha here). Vpr has been shown to induce cell death in both infected and uninfected "bystander" cells by both direct and indirect mechanisms. In chapter 2, we explored the effects of NHE1 on cell death in infected cells as a model for direct effect. Vpr-mediated bystander cell death has been postulated to occur through secreted factor(s) released by the infected target cells. Vpr protein itself in soluble form has been cited as one possibility. However, more often, though, the infected target being immune cells suggests secreted factors such as cytokine, chemokines, and neurotoxins could play an important role in bystander cell death. In Specific Aim 1, we explored the differential regulation of host cellular genes. There, we observed the gene TNF alpha as possessing a promoter motif similar to that in other Vpr-modulated genes. We therefore hypothesized that TNF alpha could be one of the secreted cytokines regulated by Vpr and able to induce cell death in bystander cells. Here, we propose to explore that hypothesis in determining whether Vpr mediates TNF alpha secretion and whether Vpr-mediated dysregulation of this cytokine induces bystander cell death. In this section, macrophages infected with virus are used as the main source of TNF alpha. Macrophages present the earliest target cell for HIV and remain the target for R5 trophic virus and a potential virus reservoir (Deng 1996, Aquaro, 2002). Macrophages produce numerous factors (cytokines/chemokines) which regulate the replication and functional events of T cells. Since bystander cell death occurs mainly in uninfected T cells (Alimonti, 2004, Holm, 2005), the immediately implicated player would be macrophages as responsible for producing factor(s) causing T cells death (Yang, 2003).

Role of TNF alpha in Immune Function

Due to its involvement in many diseases, both infectious and autoimmune, tumor necrosis factor alpha is a well studied factor in host immune defense. TNF alpha is best known as a

"pro-inflammatory" cytokine (Warren, 1990). It is produced mainly by macrophages/monocytes, natural killer (NK) cells, B cells, and TH1 polarized lymphocytes. TNF alpha is also produced to a lesser extent, by keratinocytes, neutrophils, astrocytes, endothelial cells, smooth muscle cells, mast cells, fibroblasts, and mesangial cells (kidney). TNF alpha is synthesized as a large 233 amino acid precursor, which is then cleaved and trimerizes into its active form (Baldwin, 1996, Tang, 1996). Both a membrane bound and a soluble form exist, though the majority of endocrine effects are achieved through the soluble form. Upon binding its ligand, the TNF-receptor (TNF-RI or TNF-RII), TNF may potentially activate two overlapping pathways, inflammation and apoptosis (Barnhart, 2003). Induction of an inflammatory response is the most well known of TNF's effects, and is important in anti-microbial and anti-viral immunity. TNF alpha, as a mediator of the inflammatory response, induces cytokine production, T cell activation, and immune function, modulates endothelial cell function, allowing increased perfusion of injured areas (Nawroth, 1986, Pober, 1987). Additionally, TNF is also known to mediate apoptosis by signaling through death domain containing signaling molecules TRADD and FADD, followed by activation of caspase 8 and caspase 3 (Depuydt, 2005, Yamashita, 1999). TNF alpha mediated cell death was the first identified role of TNF alpha in tumor cell death. Subsequent use of TNF as an anti-tumor agent has been hampered by the toxic inflammatory side effects of systemic TNF.

Bystander Cell Death in HIV

HIV-1 targets different cell types of the immune system, including immature dendritic cells (DC), Langerhans cells and resting T cells (Horvat, 1989, Granelli-Piperno, 1998, Ganesh, 2003). Irrespective of the presence of viral antigens in infected antigen presenting cells (APCs), the immune response eventually fails to control HIV-1 disease progression. One potential explanation for the loss of immune function is loss of T cell function due to induction of anergy or apoptosis of naive T cells thereby contributing to the early loss of CD4+ and CD8+ T cells. HIV-1 has also developed strategies to trigger apoptotic signaling in both infected and uninfected bystander cells to disarm the host immune response. In draining lymph nodes of HIV-1 infected individuals apoptosis is evident primarily in uninfected bystander cells, mainly T and B cells providing important clues for the existence of an indirect mechanism of cell death in HIV-1

infected subjects (Finkel, 1995). Apoptosis of uninfected T cells could be due to CD4 cross-linking, secretion of viral proteins and/or pro-apoptotic cytokines from infected cells (DC and T cells) as well as through cell-cell contact followed by activation

Several HIV-1 proteins have been implicated in host cell apoptosis. For instance, as discussed in Chapter 1.2.6, Nef can trigger apoptosis of bystander cells and sensitize CTLs to CD95 restricted apoptosis by activation induced cell death (Okada, 1997, Zauli, 1999). Nef is also known to trigger the initiation of apoptosis by functional up-regulation of FasL on the surface of the infected cells and by modulation of caspase dependent activation of death signaling events (Xu, 1999). The envelope glycoprotein complex (gp120-gp41) causes apoptosis in both infected and uninfected cells, is one of the main apoptosis-inducing molecules encoded by HIV-1genome (Zagury, 1993, Lu, 1994). Env that is expressed on the plasma membrane of infected cells can interact with a CD4 molecule and a suitable co-receptor to trigger cell-to-cell fusion: the resulting syncytia subsequently undergo apoptosis (Laurent-Crawford, 1993, Ferri, 2000). Similarly, other proteins such as Tat, Vpr and Vpu are also causes apoptosis using several intrinsic and extrinsic pathways (reviewed in Trono, 1995). Vpr has been associated with cell death in infected cells, potentially occurring through caspase induction, compromise of the mitochondrial membrane, induction of DNA repair, or other mechanism(s) (as reviewed in Section 1.2.9). Of interest here, Vpr has also been implicated in the apoptotic death of uninfected bystander cells (reviewed in Azad, 2000, Tungaturthi, 2003). In our laboratory, we have seen the ability of Vpr (Cy labeled) to transverse the plasma membrane. Thus, free Vpr protein in the host may be taken up into uninfected cells and cause cellular events, such as apoptosis. Such a mechanism has been implicated not only in lymphocyte cell death, but potentially in neuronal cell death associated with HIV dementia as well (Burdo, 2004). While free Vpr presents a potential source of direct HIV-induced cell death, it is unlikely to account for the massive apoptosis occurring during HIV infection. Thus, several studies, including our own, have postulated in their discussions that a soluble factor may be involved (Shi, 1998, Yang, 2003). Based on preliminary data attained in our laboratory implicating Vpr in upregulation if TNF alpha production in dendritic cells, as well as the distinct promoter motifs predicting Vpr mediated upregulation found in Chapter Two of this study, we chose to further study the effects of Vpr on TNF alpha regulation.

4.2 MATERIALS AND METHODS

Macrophage Isolation and Culture

Normal human donor blood was attained from the Central Blood Bank of Pittsburgh, PA devoid of patient identifying information using appropriate IRB approval from University of Pittsburgh. Total PBMCs were isolated using Ficoll-Histopaque density gradient centrifugation. Monocyte derived macrophages were obtained from total PBMCs through isolation of monocytes by adherence to plastic and subsequent cytokine mediated maturation to macrophages. Briefly, following Ficoll gradient centrifugation PBMCs were washed twice in 1X phosphate buffered saline (PBS), and were resuspended in DMEM without supplementation. PBMCs were plated onto 6-well or 100mm tissue culture dishes (Falcon) at 3 million or 15 million cells respectively. As approximately 10% of total PBMCs are monocytes, this yielded approximately 300,000 monocytes per 6-well plate well and 1.5 million monocytes per 100mm dish. Monocytes were allowed to adhere for 1.5-2.0 hours, followed by two washes with 1X PBS to remove un-adhered lymphocytes. Monocytes were matured into macrophages through culturing for 7-9 days in DMEM supplemented with GM-CSF (500U/ml) and M-CSF (15ng/ml). Fresh media containing the appropriate cytokines was added every 3 days by replacing half of the spent media with the new media. Maturation was followed visually by microscope. Macrophages were considered mature when they had attained characteristic macrophage morphology, including cell size 5-10 times original monocyte size and large prominent central nucleus. Following maturation, macrophages continued to be maintained in DMEM supplemented with GM-CSF and M-CSF as above through infection and stimulation.

Virus Production

HIV*vpr*+ and HIV*vpr*- were attained through transfection of proviral constructs in HEK 293T cells. The proviral constructs pnl43 *env*- and pnl43*env*-*vpr*- used in this study were obtained from NIH AIDS Reference and Reagents Program (NIH-ARP), donated by Dr. Landau. The pnl43*env*-*vpr*- construct contains a frame-shift mutation in vpr coding sequence, causing the

protein to be truncated at the 21st amino acid. Due to the instability of the 21 amino acid Vpr polypeptide, no Vpr protein is produced (as assessed by western blot in our laboratory). For transfection, HEK293T cells were plated at 25% confluence on 100mm dishes and allowed to grow overnight to reach approximately 50-60% confluence. Cells were then transfected using the calcium phosphate method as described in Majumder et. al (2005). In a 2ml polystyrene tube, sterile distilled water was added, followed by 15µg proviral DNA, as above, 5µg VSV-G envelope construct, and 50µl 2.5M calcium chloride. This mixture, totaling to 500µl, was mixed thoroughly. An equal amount (500ul) of 2X BES was then added and mixed by gently tapping the tube. The mixture was allowed to incubate at room temperature 30 minutes. Following incubation, the mixture was added dropwise to the HEK293T plates containing 8-10ml medium. After 14 hours, the medium containing the transfection mixture was removed, the cells were washed once with 1XPBS. DMEM supplemented with 10% FBS and 1% L-glutamine was added (12-15ml per 100mm plate) and cells were incubated three-five days depending on cell death. Virus supernatant was collected at 72 and 120 hours and centrifuged to remove cell debris. Infectious particle titer was determined using the reporter cell line TZM, available through the NIH-ARP. This cell line contains a copy of the beta-galactosidase gene under the control of the HIV-1 LTR, thus serving as an indicator of viral infection and replication. Virus stocks were stored aliquotted at –80˚ C degree for further use.

In order to assess the effect of virion associated Vpr, virus was inactivated using AT-2 (2'2'-Dithiodipyridine purchased from Sigma Chemicals, St. Louis, MO) as described by Rossio et. al. (1998). Briefly, virus stocks (supernatants) were treated with 250µM 2'2'-Dithiodipyridine for one hour in a rocking 37°C water bath. All virus stocks were further concentrated by ultra centrifugation at 22,000 rpm and 4°C for 1 hour. Virus pellets were dissolved in PBS and stored at 80˚C for subsequent assays.

TNF alpha Production by ELISA

Macrophages were plated at 300,000 cells/well in a 6 well dish and matured and maintained as described above. Following maturation, macrophages were infected with HIVvpr+ or HIVvpr- at an MOI of 1.5. Seventy-two hours post-infection, media was removed and cells were washed once with 1X PBS. Fresh medium was added for stimulation and LPS was added at a concentration of 100mM. To maintain consistency throughout the experiments

(N=24), all 24 sets of donor macrophages were infected with the same two batches of HIV*vpr*+ and HIV*vpr*- and treated from the same lot of lipopolysaccharide (LPS). Twenty-four hours post stimulation with LPS, supernatant was collected for measurement of soluble TNF alpha by ELISA and cell pellets were collected for TNF alpha RNA assessment, as described below. Soluble TNF alpha in the culture supernatant was measured using the ELISA kit following the manufacturer's instructions (BD Biosciences, CA).

Real-time RT-PCR to assess TNF alpha transcript level

Cell pellets from macrophages infected and stimulated for the above described experiment were collected by scraping them in 5 ml cold PBS. Cell pellets were collected by centrifugation at 1200rpm for 5 minutes. When necessary, cell pellets were flash frozen in liquid nitrogen and stored at –80˚C. RNA was extracted using the Qiagen RNeasy kit as per manufacturer's instructions (Qiagen, CA). The optional on-column DNase digestion was included to minimize contamination by genomic DNA (RNase free Dnase kit, Qiagen). RNA integrity and concentration were assessed by spectrophotmetry and agarose gel electrophoresis. RNA was then reverse transcribed to cDNA using the Taqman Reverse Transcription Reagents kit. For each reaction (to be carried out in triplicate), 1µg of RNA was reverse transcribed in a 10ul total reaction. Reverse transcription was carried out using the manufacturer's suggested cycling conditions: 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, followed by a 4°C Hold when necessary. Real-time PCR reactions were then carried out using Taqman Universal master mix and primers and probes from the Assays-On-Demand program, both from Applied Biosystems. Real-time PCR reactions were 20µl total, comprised of; 10µl 2X Taqman Universal Master Mix without UNG, 1µl 20X primer/probe mix, 0.33µg cDNA, and water. To minimize pipetting errors and deviation between samples, master mixes were created for each probe and RNA (cDNA) sample. Cycling was carried out on an ABI PRISM 7000 thermocycler, using the manufacturer's default conditions; 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Collected data were analyzed as a Relative Quantitation Study using ABI PRISM 7000 Sequence Detection Software v1.1. This analysis uses the comparative or ΔΔCT method to generate relative ratios. Prior to analysis, data were manually examined for quality control (abhorrent signals were omitted). In most cases, automatic threshold calculation and automatic baseline were utilized. Where necessary, manual

threshold was determined to be that which gave a line in the linear portion of the amplification plot and minimized the standard error in the greatest number of triplicate samples. Reactions carried out in triplicate with a standard error of >0.1 were not used. This stringent cutoff, 5 times lower than the 0.5 level suggested by the manufacturer, allowed small differences (≤2 fold) to be reported with a higher confidence. A 95% confidence interval was used to determine whether observed differences in ratios were statistically significant. Ratios are reported as the average relative to the HIVvpr- control, thus representing the magnitude of Vpr effect. In each experiment, the endogenous control RPLPO (human large ribosomal protein) was included to control for fluctuations in RNA integrity and pipetting errors. All values are therefore calibrated to RPLPO prior to expression relative to HIVvpr-. Error bars represent standard error as determined by the SDS Detection software and are generated as a measure of the differences between samples run in triplicate. CT represents the threshold cycle from which relative ratio was determined.

P24 Antigen ELISA

In order to compare the viral replication in HIVvpr- and HIVvpr+ infected macrophages, p24 antigen level was measured by ELISA. Supernatants collected at 72 hours post infection were frozen at –80˚C for storage. For ELISA, samples were thawed slowly on ice and p24 antigen levels assessed using the HIV-1 p24 Antigen Capture Assay Kit obtained from the AIDS Vaccine Program, SAIC-Frederick as per manufacturer's instructions. Briefly, supernatant samples were lysed with 10% TritonX-100 for one hour. During that time, the precoated p24 monoclonal antibody plates were washed ten times with the included Wash Solution. Following lysis, 100µL of standards and samples were added to the antibody coated plates and incubated at 37°C for 2 hours. Plates were washed in Wash solution and polyclonal anti-p24 was added diluted in Primary Antibody diluent (1:400; RPMI with 10% FBS and 2% NMS) for 1 hour at 37°C. Plates were washed ten times and secondary detection antibody, goat anti-rabbit IgG (H+L)-peroxidase labeled) was added in diluent (1:300; 2% NMS, 5% NGS, 0.01% Tween 20 in RPMI) for 1 hour at 37°C. The plates were washed ten times, followed by the addition of TMB substrate for 30 minutes to develop color. The reaction was stopped by addition of 1M HCL and read at 450nm on a colormetric plate reader.

Genomic DNA Extraction

Cell pellets (approximately $5x10^6$ cells) were collected from the PBMCs of each donor prior to macrophage adherence. Cell pellets were collected by centrifugation for 5 minutes at 1400rpm in a Sorvall RT7 centrifuge. Cells pellets were washed once with 1X PBS and stored at -20 C. Genomic DNA (gDNA) was extracted using the GFX Genomic Blood DNA purification kit (GE Healthcare, NJ) as per manufacturer's protocol. Briefly, cell pellets were thawed and excess supernatant carefully removed via pipetting. The pellet was then resuspended in the remaining unremovable supernatant (20-50µl) via vigorous vortexing. Before the cells had settled, 500µl Extraction solution was added to each tube. The solution was vortexed and then added to an included GFX column. Columns were centrifuged at 8,000rpm in a Sorvall Biofuge Pico, followed two washes, the first in Extraction solution and the second in Wash solution. DNA was eluted in 200µl distilled, autoclaved water pre-heated to 70°C. gDNA was stored at 4°C for later use.

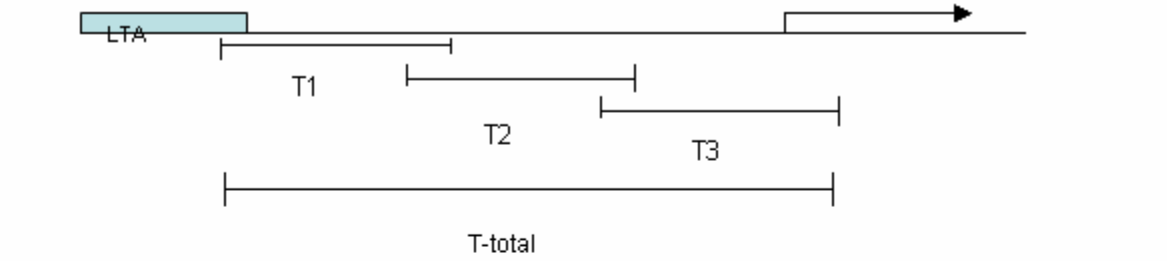
Amplification of TNF alpha promoter by PCR

In order to determine whether polymorphisms in the promoter of TNF alpha are controlling the patient-specific responses seen by ELISA, we analyzed the promoter region of each donor. The promoter region was amplified for analysis as follows. The consensus sequence of the TNF alpha promoter was attained from the NCBI nucleotide databank, clone M16441. The start of transcription was located to position 4095 in this clone, and the start of translation at 4275. In order to account for the degenerate signal attained at the ends of PCR fragments, the amplicon designated as the promoter was designed to overlap into the upstream lymphotoxin A (LTA) gene and the downstream start of transcription for the TNF alpha gene. In this way, we could assure accurate polymorphism detection and sequencing of the promoter region within this amplicon.

Denaturing High Performance Liquid Chromatography, DHPLC, is a highly sensitive means of detecting polymorphisms in the promoter. Using chromatography column separation, single nucleotide changes can be detected in a high-throughput 96-well format. This technique was chosen initially for analysis of the TNF alpha promoter as it gave us the opportunity to screen both the donor samples used for ELISA analysis in this study, as well as large numbers of normal donors from which to generate background polymorphism data and percentages. For

DHPLC analysis, the TNF alpha promoter region was divided into three regions. Amplicons were designed within the following parameters; 1) Optimal DHPLC amplicons should be 700 base pairs maximum, 2) Primers should overlap by ≥50 base pairs, 3) Within amplicons, the melt temperature should be consistent, not varying more than three degrees. (Xiao, 2001 and Dr. J. Martinson, personal communication). The TNF alpha promoter amplicon melt temperature was predicted using the DHPLC Melt Program available at http://insertion.stanford.edu. Based on the melt profile, six primers were designed in order to amplify three regions of the TNF alpha promoter, T1-T3. A pictorial representation is included below (Figure 12) as well as a table indicating the primer sequence, nucleotide position # in the GenBank sequence M16441, and amplicon length.

For sequencing analysis, the same six primers were used and T-total, comprising the entire TNF promoter amplicon, with the desired overlap of the LTA and TNF transcribed areas was also generated as depicted in Figure 12. For DHPLC analysis, PCR reaction was carried out using AmpliTaq (Applied Biosystems) polymerase. Following testing of both temperature and magnesium gradients, optimal TM for all primer sets was found to be 54 degrees Celsius and optimal magnesium concentration 2.5mM. Final 27ul reactions were comprised of; 2.5ul 10X AmpliTaq Buffer, 19.125ul sterile distilled water, 12.5ul 25mM MgSO4, 0.5ul primer mix (forward and reverse primers at a final concentration of 10pmol/ul), 0.25ul dNTP mix, 0.125ul AmpliTaq, and 2ul gDNA extracted as above. For sequencing, PCR was a carried out using Promega PCR Master Mix (Promega Life Sciences). Final 20ul reaction consisted of 10ul 2X PCR master mix, 2ul forward primer 10pmol/ul, 2ul reverse primer 10pmol/ul, 3ul sterile distilled water, and 3ul gDNA extracted as above. As total genomic DNA was utilized as template, touch down PCR was utilized in order to minimize misprimed, nonspecific amplification products. Final cycling conditions were; Step 1) 95°C for 2 min., 2) 95°C for 30 sec., 3) 61°C for 30 sec., decrease at -0.5°C per cycle, 4) 72°C for 45 sec., 5) Repeat steps 2-4 for 15 cycles, 6) 95°C for 30 sec., 7) 54°C for 30 sec., 8) 72°C for 45 sec., 9) Repeat steps 6-8 for 21 cycles. Amplification was confirmed by subjecting PCR products to electrophoresis on a 1.0% agarose gel with Ethidium bromide added. Amplicon sizes are indicated on Figure 12B.



Construct	Forward primer	Reverse primer	Amplicon size
T1	T1F 2767-2787 5' CGAAAGGCTCTGAAAGCCAGC-3'	T1R 3317-3294 5'TAAACGTCCCCTGTATTCCATACC-3'	551 bp
T2	T2F 3191-3215 5'GAGAATGTCCAGGGCTATGAAAGT-3'	T2R 3834-3811 5'GCCACTGACTGATTTGTGTGTAGG-3'	644 bp
T3	T3F 3742-3762 5'CCTGGTCCCCAAAAGAAATGG-3'	T3R 4377-4359 5'GGCTGAGGAACAAGCACCG-3'	636 bp
T-total	T1F 2767-2787 5' CGAAAGGCTCTGAAAGCCAGC-3'	T3R 4377-4359 5'GGCTGAGGAACAAGCACCG-3'	1610 bp

Figure 12: Amplification of the TNF promoter region for analysis of polymorphism.
The TNF promoter region is limited by the upstream presence of the Lymphotoxin A gene (LTA). T1, T2, and T3 represent the regions used for DHPLC analysis. T-total is the amplicon of the entire TNF promoter, used for sequence analysis. Primers and amplicon length are denoted in the Table which follows. The numbers in the primer name refer to where it binds in the GenBank M16441 sequence from which primers were designed.

Analysis of Polymorphism: DHPLC/Sequencing

DHPLC was chosen as a high throughput method of screening for polymorphisms in the TNF alpha promoter region. For DHPLC analysis, PCR products attained as above were melted and allowed to form heteroduplexes. Optimal DHPLC temperature for amplicons T1-T3 was determined by running a gradient of one degree increments from 59-63°C. An optimal temperature was considered to be that at which the peak for the amplicon was maximally resolved prior to breakdown. Optimal temperatures were T1-61°C, T2-61°C and T3-63°C.

The extensive polymorphism found in the TNF alpha promoter by DHPLC analysis necessitated the use of sequencing to determine the existence of individual single nucleotide polymorphisms (SNPs). For sequencing, T-total region was amplified as specified above. As maximal readable sequence in a sequence run from the University of Pittsburgh sequencing core

facility averages 500 base pairs, the promoter was sequenced in regions T1-T3 as per DHPLC analysis. PCR was carried out as specific above and sample cleanup was carried out prior to sequencing via treatment with SAP (shrimp alkaline phosphatase) and Exo I nuclease (Exonuclease I). Exo-SAP reaction was as follows for a 20ul total reaction; 1.0ul 10X SAP Buffer, 1.0µl SAP (1U, Roche), 0.05µl Exo I (0.5U, New England Biolabs), 7.95µl sterile distilled water, 10µl PCR product. Samples were incubated for 60 minutes at 37°C, followed by denaturation at 85°C for 15 minutes. Samples were stored at this point at 4°C when necessary. Sequencing was then carried out using the method of automated fluorescent dideoxy nucleotide sequencing. The BigDye sequencing reaction kit (Applied Biosystems) was used in a 10µl total reaction as follows; 0.6µl BigDye Ready Reaction Premix, 1.7µl 5X BigDye Sequencing Buffer, 2.0µl primer (2pmol/µl), 0.7µl sterile distilled water, 5µl Exo-SAP treated PCR product. Cycling was as follows, Step; 1) 96°C for 1 min., 2) 96°C 10 sec., 3) 50°C for 5 sec., 4) 60°C for 4 min., 5) Repeat step 2-4 for 25 cycles. Unincorporated dye terminators were removed via Ethanol/EDTA precipitation. To each 10µl sequencing reaction, 5µl 125mM EDTA and 60µl 100% ethanol were added. Samples were inverted approximately four times to mix, incubated at room temperature 15 minutes, followed by centrifugation at 2500xg for 30 minutes at 4°C. Supernatant was removed and sampled were washed by addition of 60µl 70% ethanol and centrifugation at 1650xg for 15 minutes at 4°C. Supernatant was removed and remaining alcohol was allowed to evaporate for 10 minutes at room temperature. The plate was then sealed and delivered to the Genomics and Proteomics Core Laboratories of the University of Pittsburgh, where sequencing was carried out on an ABI 3730. The resulting sequences were viewed and manually verified using Chromos v2.3 (http://www.technelysium.com.au/chromas.html). Alignments were carried out using Sequencher (GenCodes Corporation). To construct each donor promoter region, sequences from regions T1-T3 forward and reverse reactions (6 reactions total) were aligned to create a donor consensus sequence. Donor consensus sequences were then aligned and single nucleotide polymorphisms were mapped. Of note, six deviations from the GenBank M16441 sequence were noted. For all deviations, the same base was seen in all samples tested as well as all of a set of 20 patient samples sequenced as part of another study, indicating a significant deviation from the GenBank sequence.

Apoptosis staining and flow cytometry

Analysis of apoptosis was carried out using the Apoptosis Detection kit (BD Biosciences, San Diego, CA) as per the manufacturer's instructions. Briefly, macrophages were infected with HIV*vpr*+ or HIV*vpr*- virus and stimulated with LPS for 24 hours. Supernatant containing TNF alpha was incubated with healthy donor peripheral blood lymphocytes (PBLs) for 24 hours. At the end of treatment cells were washed twice with cold FACS buffer and analyzed for apoptosis by flow cytometry. To detect apoptosis, cells were resuspended in 100µL sterile binding buffer containing 10mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5mM CaCl2, incubated with Annexin V-FITC and PI (propidium iodide) for 15 minutes at RT in the dark and analyzed by flow cytometry. The percentage of Annexin V-FITC and PI positive cells were estimated by Cell Quest software.

4.3 RESULTS

Vpr Induces host-specific upregulation of TNF alpha in response to LPS in infected cells

As macrophages represent relevant in vivo early targets for HIV-1 infection as well as major producers of TNF alpha, here we undertook a study of the effect of Vpr on TNF alpha production from macrophages (Balter, 1996, Scarlatti, 1997). In order to determine whether infection with HIV*vpr*+ causes dysregulation of TNF alpha, macrophages were cultured and infected with HIV*vpr*+ and HIV*vpr*-. TNF alpha secretion in response to LPS stimulation was assessed by ELISA, yielding a ratio of response with and without the presence of Vpr. Briefly, normal donor blood was attained from the Central Blood Bank of Pittsburgh, Pennsylvania. Peripheral Blood Mononuclear cells (PBMCs) were isolated through gradient density centrifugation and monocytes were separated by adhesion to plastic. Monocytes were matured into macrophages through incubation for 7-9 days in medium containing GM-CSF and MCSF. Maturation was followed visually until cells attained characteristic macrophage morphology at 7-9 days. Following maturation, cells were infected with HIVvpr+ or HIVvpr- virus as described in Methods. Three days post-infection (peak infection day as assessed by flow cytometry for p24

antigen), cells were washed and given fresh medium containing 100mM lipopolysaccharisde (LPS). Twenty-four hours post stimulation, cell supernatant was collected and assessed for soluble p24 antigen by ELISA. As controls in the first experiments, supernatant was also collected pre-stimulation from untreated, and HIVvpr+ and HIVvpr- virus infected cells. In all three pre-stimulation samples, over the course of three donors, TNF was undetectable. Therefore, in the remaining donors tested, these pre-stimulation samples were not included. Results of the ELISA are presented in Figure 13 represents data derived from multiple donors (N=22). Figure 13 presents the ratio of TNF alpha secreted in response to LPS stimulation in the presence of HIV*vpr*+ versus HIV*vpr*- virus infection, thus indicating the effect of Vpr. Results indicate that the amount of TNF alpha produced as an effect of the presence of Vpr varied greatly by donor, with a range from a ratio of 1.0 to 8.5. Median response was 1.77 and the median was used to classify donors as either low responders (<1.77), medium responders (1.77-3.0) or high responders (>3.0). These classifications will be utilized in later analyses of function. Similar results were seen in three donor samples treated with CD40 ligand (CD40L), thus treatment with CD40L was not carried throughout the experiment, as the maximum number of cells attainable from a single donor proved a limiting factor. These results are mentioned here, as CD40L stimulation utilizes a separate signaling pathway from that of LPS stimulation. Thus similar results attained using CD40L and LPS may indicate that Vpr is not working through either of these molecule's unique signaling pathways, but through a shared mechanism.

The results attained in the above experiment suggest that Vpr specifically induces cytokine dysregulation, mainly upregulation of TNF alpha from infected macrophages in a donor specific manner. As indicated in the Introduction, Vpr has been shown to increase viral replication in both macrophages and T cells by increasing the HIV-1 LTR mediated transactivation (Vanitharani, 2001). While initial infection rate was found to be similar by flow cytometry (Appendix), the presence of Vpr may have subsequently increased viral replication and its subsequent cellular events. Thus the single cycle replication system was used here to rule

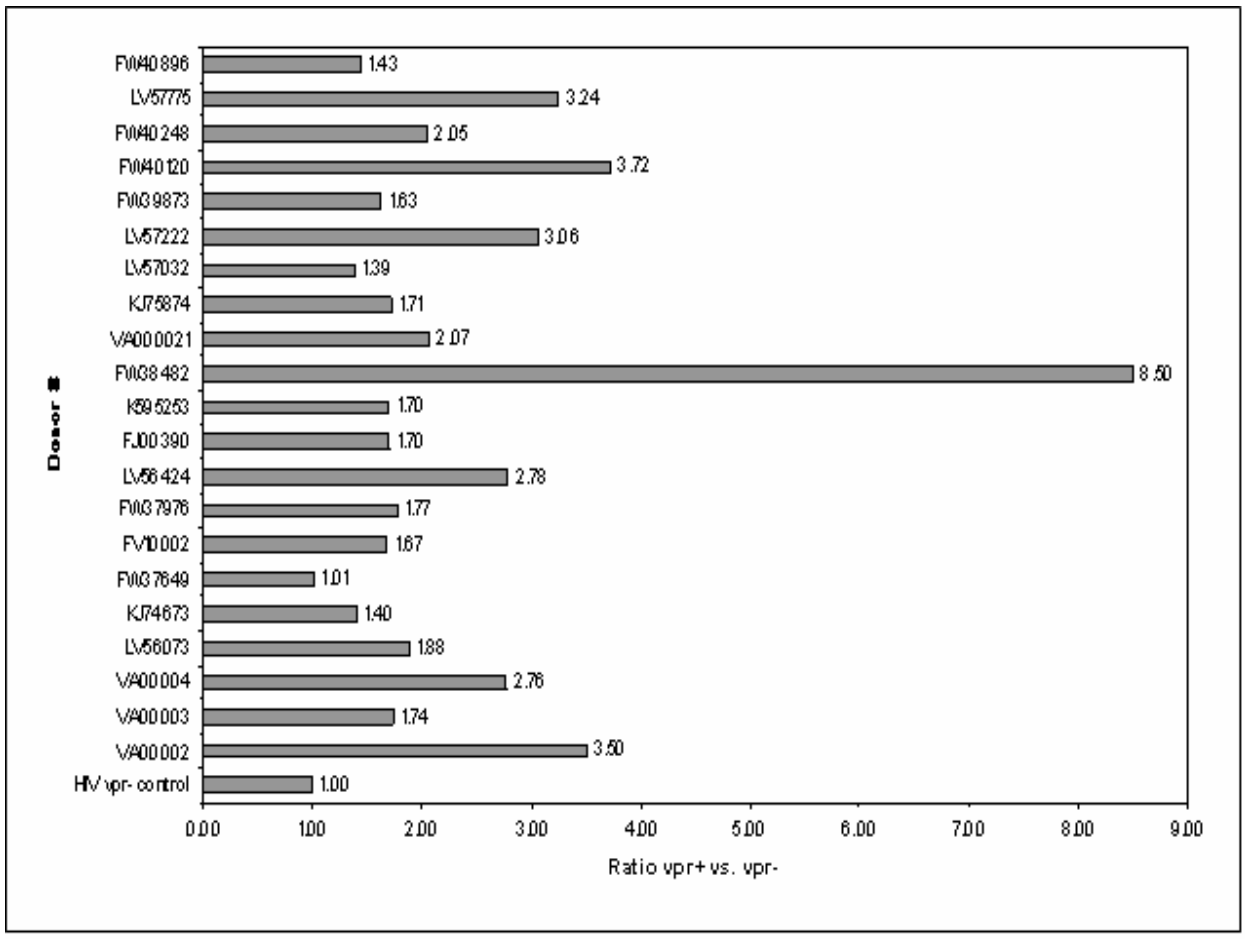


Figure 13: TNF alpha secretion from HIVvpr+ or HIVvpr- infected macrophages stimulated with LPS.

Normal donor macrophages were isolated and infected with HIVvpr+ or HIVvpr- for 72 hours, as described in Methods. Macrophages were stimulated with LPS (100mM) for 24 hours and supernatant was collected for TNF alpha ELISA. Results are presented as the ratio of TNF production in HIVvpr+ infected cells following LPS stimulation versus TNF production from HIVvpr- infected cells following LPS stimulation (HIVvpr+/HIVvpr-). Median value is 1.77. To allow for cross-referencing in later figures, the donor identification number is on the X-axis.

A

Patient ID	p24 ng/ml	TNF ratio HIVvpr+/HIVvpr-
LV57222	490	3.06
FW37976	297	1.77
FW37649	1,617	1.01
FW38482	183	8.5

B

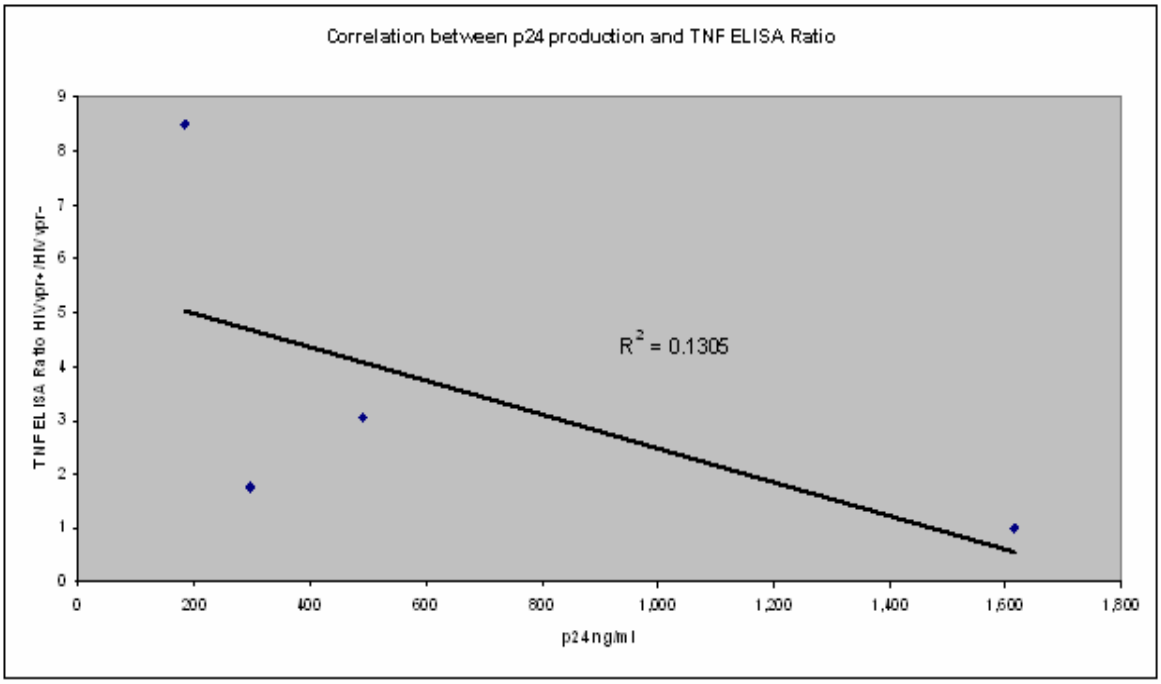


Figure 14: p24 antigen levels, as assessed by ELISA.

In order to assess the effect of virus replication on TNF response, macrophage supernatant samples collected post infection and LPS stimulation were used to test for p24 antigen level by ELISA. The table below shows p24 antigen levels and TNF production from HIVvpr+ versus HIVvpr- infected macrophages stimulated with LPS for the four donors tested. Linear regression analysis indicates that no correlation exists between virus replication, as denoted by p24 production, and TNF production in response to LPS.

out an effect of reinfection and its confounding effects. Yet, there exists the potential that increased LTR transcription and virus production may effect TNF alpha production and response. Thus, the TNF upregulation seen here may be a side effect of increased virion production and associated signaling events. In order to rule out this confounding variable, macrophage supernatants assessed above for TNF alpha were also subjected to p24 antigen ELISA. P24 antigen was used as a surrogate for virus titer due to the noninfectious nature of

Unit #	Sex	Race
LV56073	FEMALE	WHITE
KJ74673	MALE	WHITE
FW37649	FEMALE	WHITE
FV10002	MALE	WHITE
FW37976	FEMALE	AFRICAN AMER
LV56424	MALE	WHITE
FJ00390	FEMALE	WHITE
K595253	FEMALE	WHITE
FW38482	FEMALE	WHITE
VA000021	UNKNOWN	UNKNOWN
KJ75874	FEMALE	WHITE
LV57032	MALE	WHITE
LV57222	MALE	WHITE
FW39873	FEMALE	WHITE
FW40120	MALE	WHITE
FW40248	MALE	UNKNOWN
LV57775	FEMALE	WHITE
FW40896	MALE	WHITE
GP77229	FEMALE	WHITE
LV58032	MALE	WHITE
LV58330	FEMALE	WHITE
LV57475	FEMALE	WHITE
LV57482	MALE	WHITE

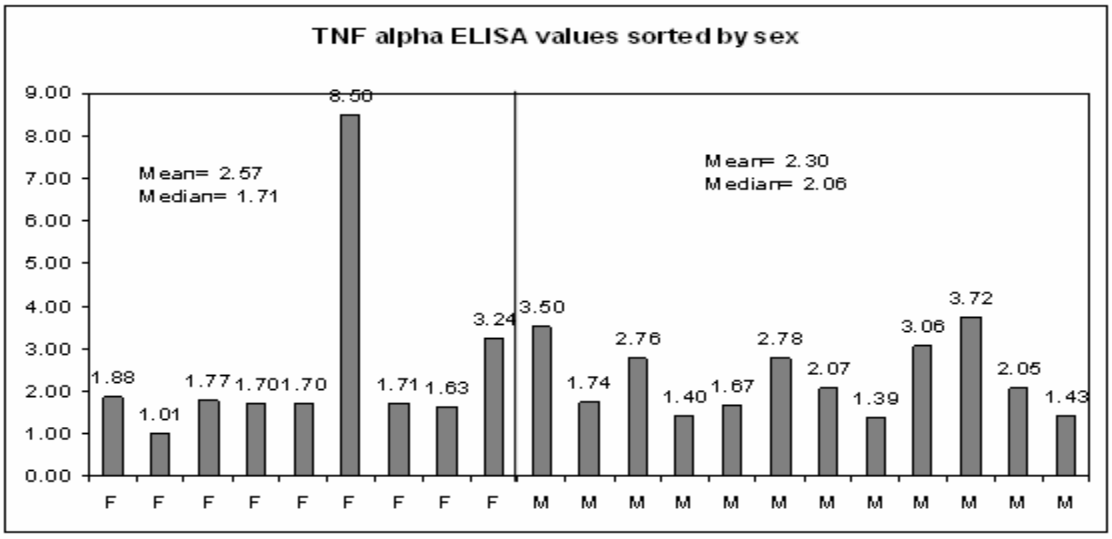


Figure 15: **Summary of donor information for normal human macrophage samples.**

Normal human blood was attained from the Central Blood Bank of Pittsburgh, Pennsylvania and used as a source of macrophages. (A) Unit # refers to the unit of blood collected and is not associated with specific donor. Sex and race were as determined by the Central Blood Bank upon donor interview. Table represents only those donors for whom age and/or sex was available. Donors for whom neither were available were removed from this analysis. (B) In order to determine whether a correlation exists between sex of donor and response to LPS stimulation as assessed by TNF ELISA, ratios (HIVvpr+/HIVvpr-) were sorted by sex of donor. Mean and Median of each group were calculated and are displayed.

particles produced in the single round system. The results presented in Figure 14 indicate that p24 antigen levels did not correlate with increased TNF alpha response to HIV*vpr*+ virus infection. While attempts were made to fir other regression lines to this data, no equation tested (linear, polynomial, logarithmic, exponential) gave an R^2 value of greater than 0.2. The lack of a correlation between p24 values and TNF alpha production suggests that the observed effect could be due to the combination of host genetic factors that regulate TNF alpha transcription (specifically the TNF alpha promoter polymorphism) and the presence of Vpr from the virus.

To understand the role of host factors in TNF alpha production, we contacted the Central Blood Bank from whom the blood samples used were obtained and further obtained donor information. Due to the confidentiality of prospective blood donors, the only attainable characteristics were race and sex. These characteristics are listed next to the Blood Unit # (designated in this study the donor ID) in Figure 15. In this study, separate races were not represented and thus race as a factor in TNF alpha response could not be assessed. Estrogen has been shown to decrease TH1, inflammatory cytokine responses, including TNF alpha. Vpr has previously been shown to bind a member of the same family as the estrogen receptor, the glucocorticoid receptor, in order to effect changes in host cellular transcription. Thus, given the equal distribution of male and female patients, we assessed whether a difference in TNF alpha response to LPS stimulation in HIVvpr+ versus HIVvpr- infected cells correlated with gender of the donor. TNF ELISA ratios (HIVvpr+/HIVvpr-) were sorted into male and female categories in the graph presented in Figure 15. For each group, the mean and median were computed and are included on the graph. No significant difference was noted in either the mean (M=2.30, F=2.57) or the median (M=2.06, F=1.71) for these groups. Thus, gender was not a determinant in Vpr-induced TNF alpha expression followed by LPS stimulation.

Production of TNF alpha followed by external stimulation varies between individuals. The ratio presented above and throughout the next set of analyses are utilized in order to correct for any differences in basal TNF alpha secretion by different donors. Difference in ratios was chosen instead of raw values, as analysis of the raw TNF ELISA data indicated that the range of basal production by donors varied greatly. I further analyzed these data in order to determine whether those donors producing a higher ratio of TNF in HIV vpr+ versus HIV vpr- samples were the same donors who might be considered “high” TNF producers (followed by LPS

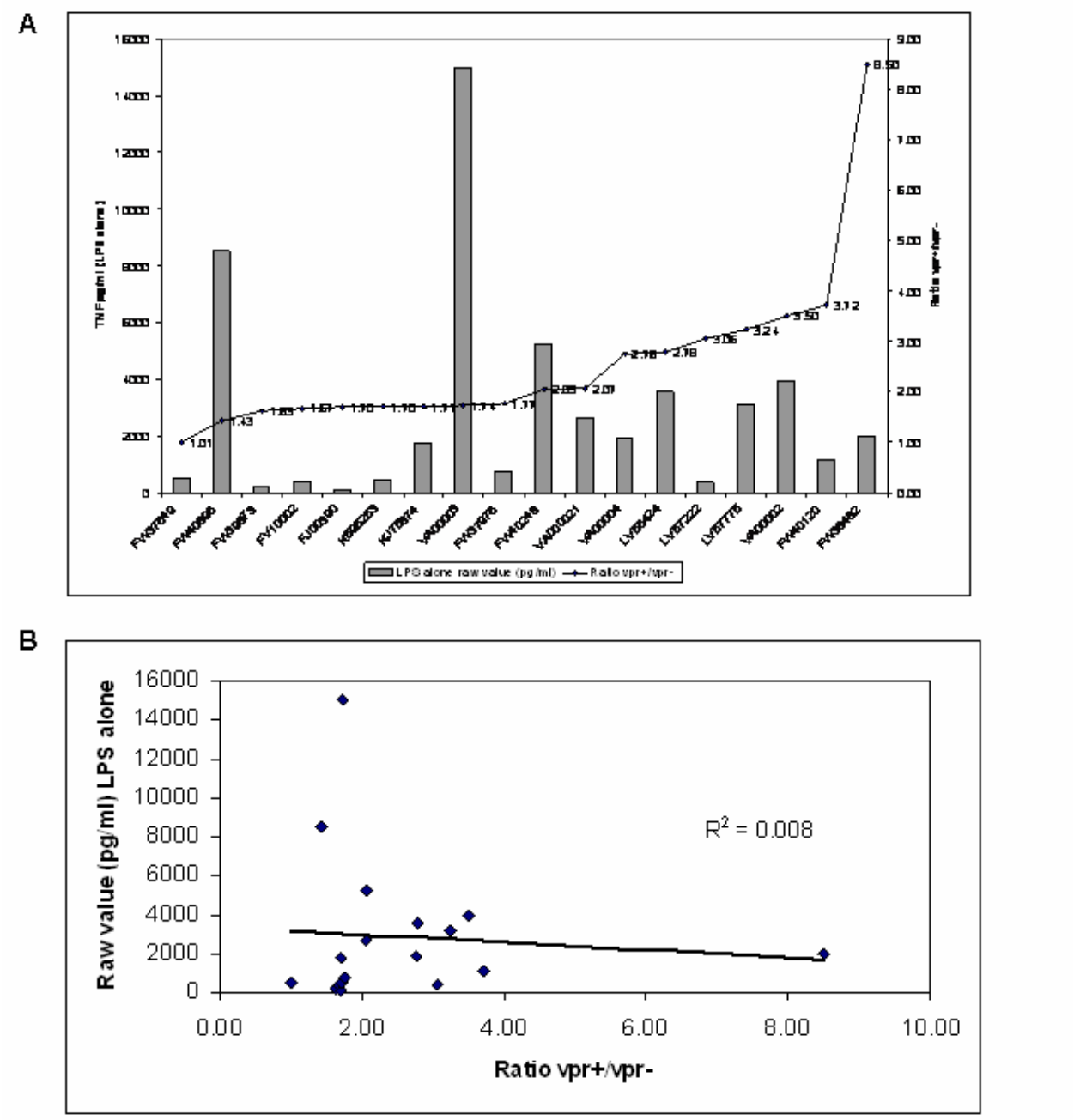


Figure 16: Comparison of raw TNF ELISA value to HIVvpr+/HIVvpr- ratio.

(A) TNF ELISA results from macrophages infected and stimulated for 24 hours with LPS as described in the previous section were graphed in order to determine whether a correlation exists between basal TNF output in response to stimulus and TNF response in HIVvpr+ versus HIVvpr- macrophages. LPS alone denotes uninfected macrophages from the same experiment exposed to LPS. HIVvpr+/HIVvpr- is the ratio of TNF alpha secreted in response to LPS stimulation in HIVvpr+ infected versus HIVvpr- infected macrophages. (B) Linear regression to determine if a correlation exists between LPS alone value and Ratio of HIVvpr+/HIVvpr-. R^2 value for the line is included

stimulation). The literature has long described differences in the levels of basal TNF alpha production in different donors. High TNF alpha production has been linked to autoimmune disease and complications in malaria and other infections (Bayley, 2004). Thus, it would be quite important to delineate whether those donors who showed an increased ratio in HIV*vpr*+ versus HIV*vpr*- were the same donors who produced high levels of TNF alpha in response to LPS stimulation alone, i.e. were these the same people who would have responded higher to any stimulus. Figure 16A is a double graph depicting the ratio of response to LPS stimulation alone (grey bars- axis at left), as compared to that in the presence of HIVvpr+ versus HIVvpr- (black line-axis at right). This allows us to separate the generally "high producing" donors from those producing high TNF alpha in response to Vpr. If the trends are the same between the bar graph and the line graph, we would assume that the donors who respond highest to Vpr would respond higher to general stimulus, thus this may not be a Vpr-specific effect. In order to ascertain this, samples are presented in order of increased response to Vpr, or increasing ratio HIV*vpr*+/HIV*vpr*-. At first glance, it is apparent from this analysis that those donors producing higher amounts of TNF alpha in response to LPS do not necessarily produce higher amounts in the presence of HIV *vpr*+ versus HIV *vpr*-. An analysis of the correlation between these two ratios was conducted and is presented in Figure 16B. This analysis confirms that no correlation exists between donor response to LPS alone and response to LPS in the presence of HIV-1 Vpr suggesting specific regulation of TNF alpha by HIV-1 Vpr.

One potential hypothesis could be that TNF alpha production in response to LPS alone versus that in the presence of Vpr may recruit different transcription factors to the TNF promoter thus altering transcription. Along this line, Vpr has been shown to bind several transcription factors, including SP1, GR, and NF-kB, all with sites within the TNF promoter, as depicted in Chapter 2, Figure 5. In order to explore this hypothesis, we will first determine whether regulation of TNF response by HIV-1 Vpr is, in fact, controlled at the transcriptional level. We will then explore the TNF alpha promoter in greater detail in the remainder of this Chapter.

Regulation of the TNF alpha response to LPS by HIV-1 Vpr is at the level of transcription

Results attained in Chapter 2 of this study indicate that Vpr is able to induce dysregulation of host genes at the level of transcription. In addition, results in Chapter 2, showed

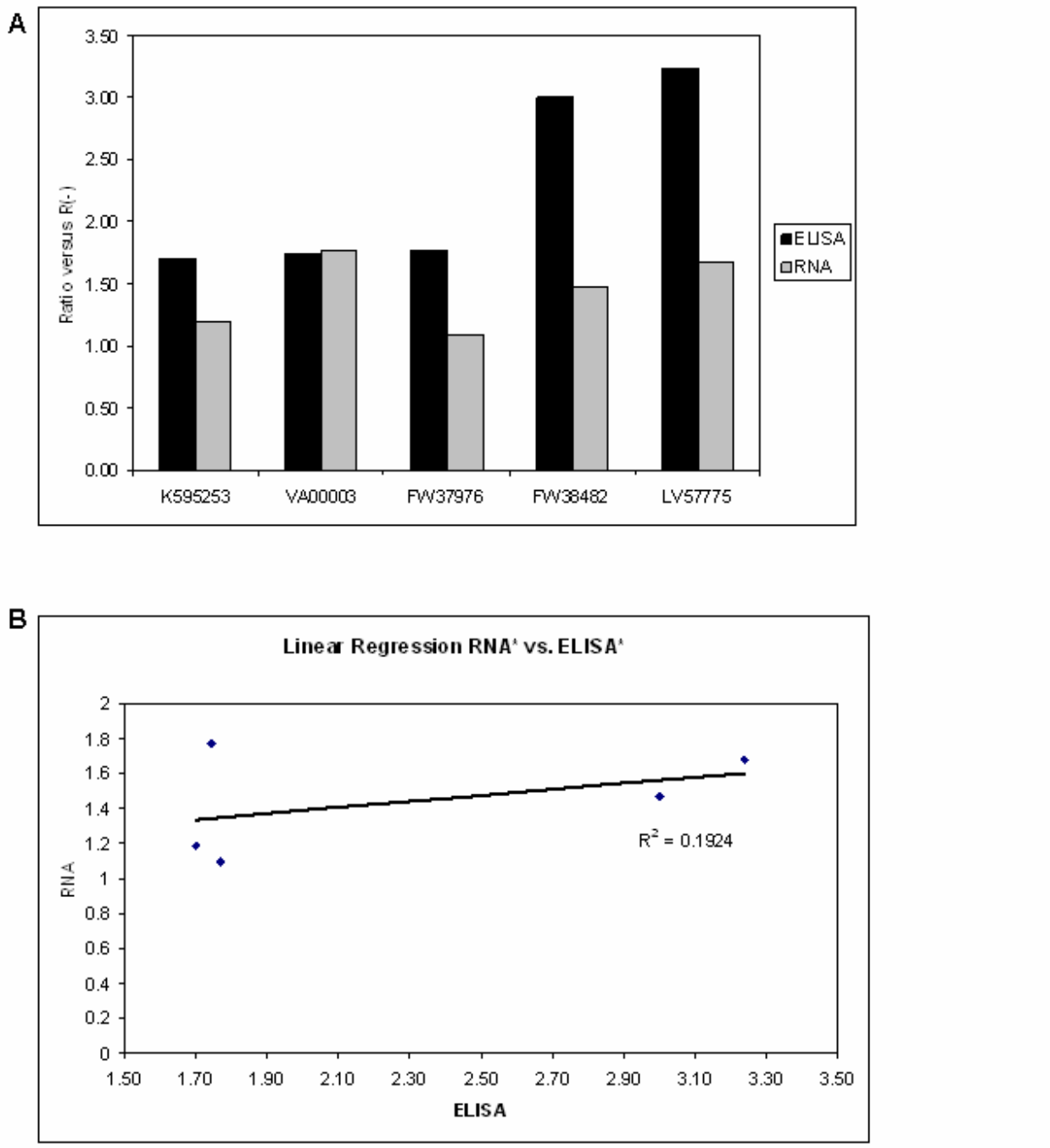


Figure 17: **TNF mRNA in HIVvpr+ and HIVvpr- infected macrophages following 24 hr LPS stimulation. Macrophages infected and stimulated for ELISA data were collected by scraping. RNA was extracted using Qiagen RNeasy kit. Real time RT-PCR was carried out using primers and probe set specific for TNF alpha or the endogenous control RPLPO. A) Comparison of TNF secretion in the supernatant of HIVvpr+ versus HIVvpr- cells as assessed by ELISA to TNF mRNA transcript ratio in the same cells (24 hours post stimulation). B) Linear regression analysis of data attained by ELISA and real time RT-PCR. R2 value for the line is included.**

that several transcription factors that are known to be bound and utilized by Vpr are present within the promoter region of TNF alpha. Taken together, these findings indicate that the regulation of TNF alpha in response to LPS stimulation in the presence of Vpr may be occurring at the transcriptional level. In this section, we studied the effect of Vpr on TNF transcription using quantitative real time RT-PCR.

In order to assess whether a correlation existed between TNF alpha production as measured by ELISA in the above experiment and TNF mRNA, initial experiments utilized the macrophages samples generated above for ELISA analysis. Macrophages were matured, infected with HIVvpr+ or HIVvpr-, and stimulated for 24 hours with 100mM LPS, as described above. Cell pellets were collected at the same time as collection of supernatant for assessment by ELISA. Following RNA extraction, reverse transcription was carried out, followed by real time RT-PCR. Primers and probes specific for TNF and the endogenous control RPLPO were attained through the Applied Biosystems Assays on Demand program. The primer and probe set for TNF alpha was designated "_m1" by the manufacturer, indicating that it has been quality assurance tested and is guaranteed not to amplify genomic DNA. This was confirmed in our laboratory using macrophage genomic DNA. Real time RT-PCR was carried out in triplicate and all values were normalized to RPLPO. Ratios were calculated via the ΔΔCT method of relative quantitation (As described in ABI User Bulletin #2, 1997) and are presented as the ratio relative to infection with HIV*vpr*-. Figure 17 shows the results attained from five donors. Interestingly, the ratios were found not to correlate with the ELISA results from the same cells (Figure 17), with an R^2 value of 019. These results were surprising and thus I searched the literature for references on TNF alpha transcriptional regulation. Published reports, which undertook time course analyses indicated that TNF transcription may occur as early as one hour post LPS stimulation (Zhu, 1994). Thus, I performed a time course to determine whether TNF alpha mRNA transcription in response to LPS stimulation was regulated at an earlier time point by HIV*vpr*+.

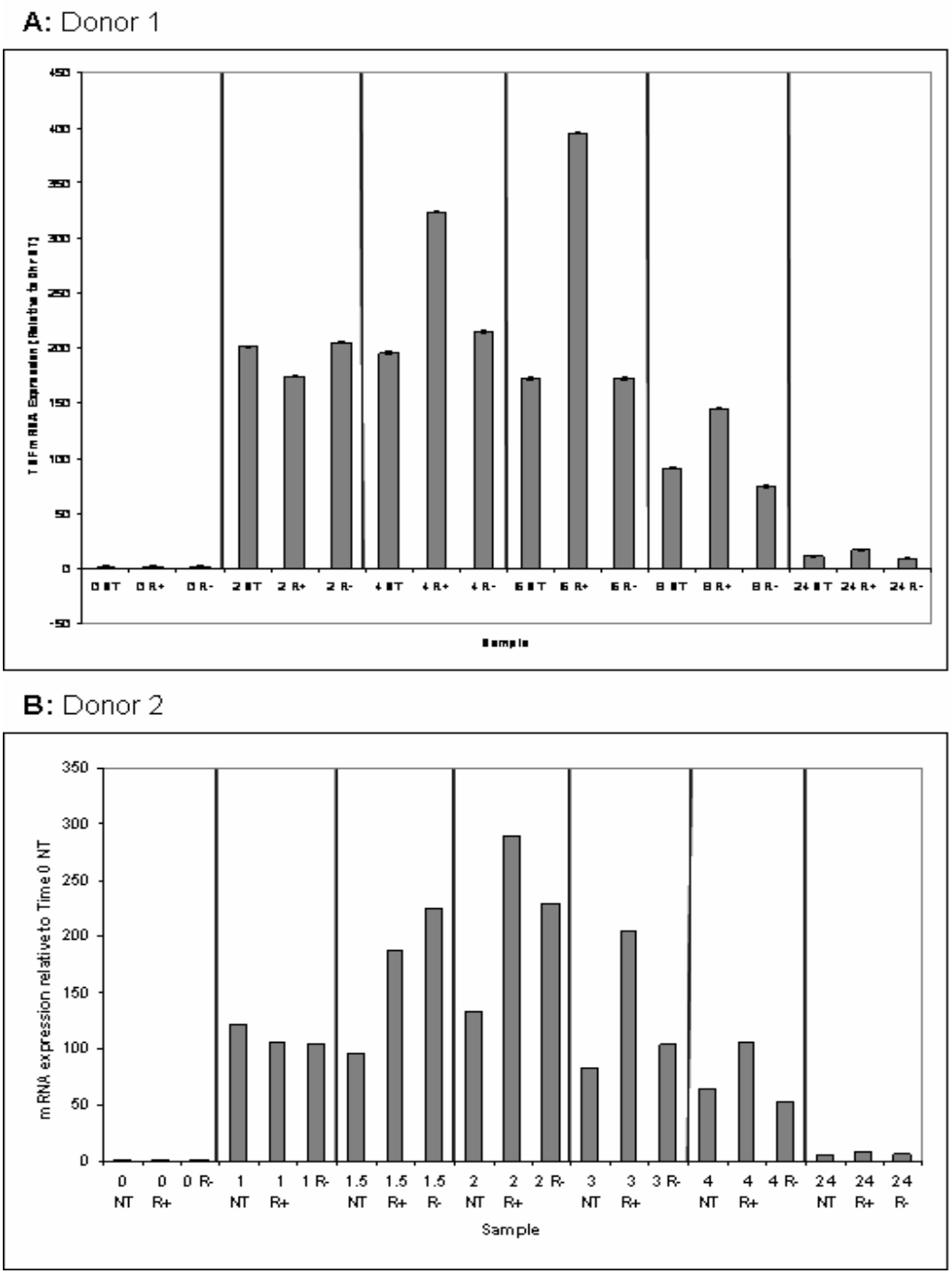


Figure 18: Timecourse analysis of TNF alpha mRNA in HIVvpr+ and HIVvpr- infected macrophages in response to LPS stimulation.

Macrophages were isolated and infected for 72 hours with HIVvpr+ or HIVvpr- as described in Methods. At Time 0, noted below, cells were exposed to 100mM LPS. Cells were collected for analysis at the times indicated post LPS-stimulation. Real time RT-PCR was carried out as described previously. Each donor A) Donor 1 and B) Donor 2, gave a different profile and are therefore presented separately.

Time course analyses of TNF alpha transcription

Macrophages were infected as described above for 72 hours with HIV*vpr*+ or HIV*vpr*-. Following infection, cells were washed and stimulated with medium containing 100mM LPS and samples (cells) were collected every 2-4 hours considering the LPS addition as time 0, as indicated in Figure 18. At 24 hours, supernatant was collected for assessment of TNF alpha secretion by ELISA. RNA extraction, reverse transcription, and real time PCR were carried out as described above for the different timepoints. All values were again normalized to the endogenous control RPLPO, followed by calibration to the Time 0 or no treatment sample. Figure 18 represents the results from two donors. All values are presented as ratio relative to Time 0, no Treatment. The heightened values of the LPS treated samples, all >100 fold higher than the no treatment sample provide internal validation of the LPS stimulation. As expected, donors differed slightly in their response to LPS. While transcription in Donor 1 peaked at six hours post stimulation, Donor 2 peaked slightly earlier at 2 hours post stimulation. Despite differences, both donors showed a similar pattern. Initial transcription of TNF alpha mRNA was similar in HIV*vpr*+ and HIV*vpr*- samples. Within approximately one hour of this initial peak, in both donors, the HIVvpr+ infected cells began to show an increased level of TNF alpha mRNA. This difference remained and slowly diminished to a minor ratio at 24 hours post stimulation. These timecourse results are in accordance with the published literature, which has cited an increase and peak in TNF alpha mRNA production anywhere from 1-6 hours post stimulation, depending on the study. In light of the results presented here in multiple donors, these discrepancies in the literature may be due to differences in the donors used for experiments. Initial TNF alpha ELISA results showed significant differences in host response to LPS stimulation and the results of our transcriptional work also indicate differences in host response, both at the levels of mRNA and soluble TNF alpha protein secretion in response to LPS stimulation. Thus, these results indicate that Vpr, in the context of infection, is able to induce an increase in TNF alpha mRNA in response to LPS stimulation, suggesting that the effect of Vpr on TNF alpha is at the transcriptional level.

Effect of Virion associated Vpr

During HIV infection in vivo, Vpr protein is available to the cell in three forms; cell-associated (de novo synthesized), virion-associated, and free Vpr protein (reviewed in Tungaturthi, 2003). The rapid and error prone replication cycle is important in conveying to HIV its ability to subvert the immune system, through rapid mutation and sequence evolution. Yet, this process also results in a large number of non-infectious virions being produced. The ratio of noninfectious to infectious virions is thought to be approximately 10,000:1 in the normal host (Rusert, 2004). Many of these noninfectious virions, though replication deficient, still carry virion associated proteins, including Vpr. Non infectious virions have also been shown to bind

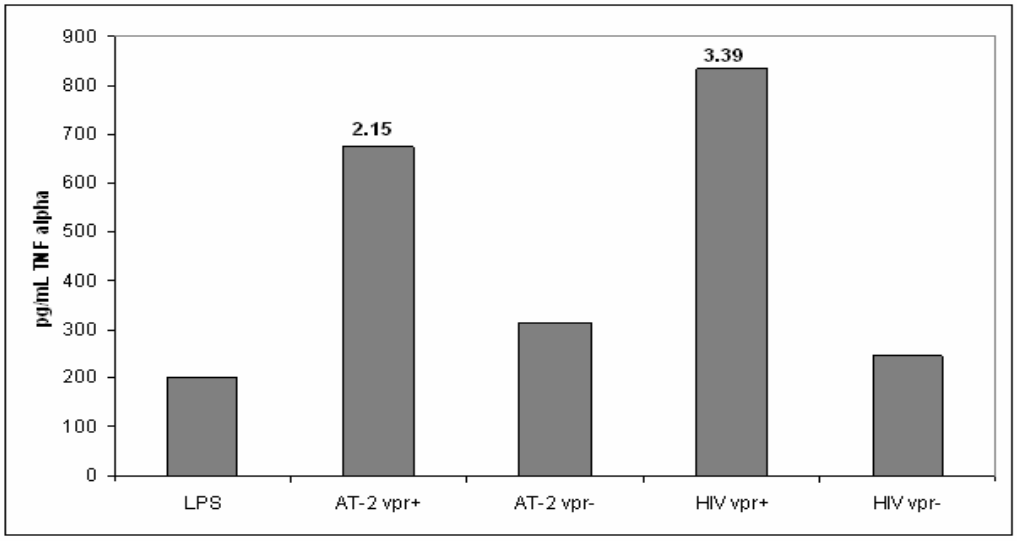


Figure 19: Determine the effect of Vpr in the absence of virus replication.

In order to assess the effect of virion associated Vpr protein, macrophages were treated with AT-2 treated virus. HIV*vpr*+ and HIV*vpr*- were inactivated with AT-2 compound via the method of Rossio (1998). Macrophages were treated with 1000ng inactivated HIV*vpr*+ or HIV*vpr*-. Seventy-two hours post treatment, supernatants from four separate plates were pooled and subjected to TNF alpha ELISA. For comparison, each donor was infected with replication competent HIV*vpr*+ and HIV*vpr*- in order to assess the potential of each donor for TNF production. Representative of experiments carried out in three donors. Number represents the ratio of HIV*vpr*+/HIV*vpr*- in either treatment.

and be internalized by several types of immune cells, including macrophages (Kornbluth, 1993, Pudney, 1994). Thus, noninfectious virions in the host may present a significant and important source of Vpr protein. We therefore wanted to determine whether virion associated Vpr, in the absence of viral replication, is sufficient to alter the TNF response to LPS stimulation. In order to carry out this experiment, we produced inactivated HIV*vpr*- and HIV*vpr*+ as described (Rossio, 1998). This method uses treatment with the chemical AT-2 to render virions noninfectious after fusion and entry. Normal donor macrophages were treated with 1000 ng p24 equivalent of AT-2 treated HIV*vpr*+ or HIV*vpr*- virus. For each donor used, in addition to the duplicate samples used in previous experiments, samples were repeated in quadruplicate and pooled prior to TNF alpha measurement. This was to compensate for small differences which may occur due to the less accurate method of virion measurement utilized here, p24 antigen ELISA measurement, as well as differences in the number of virions taken up by macrophages. In addition, as donor ratios are expected to differ, thus an assessment of whether or not noninfectious particles have an effect would be dependent on whether the donor was expected to respond to infectious particles. Thus, alongside the treatment with noninfectious particles, each donor was also treated with infectious HIV*vpr*- and HIV*vpr*+ similar to previous experiments. TNF alpha production was measured and the results are presented in Figure 19. The donor shown would be expected to show a ratio of 3.39 HIV*vpr*+/HIV*vpr*-, as evidenced by the infection with HIVvpr+ and HIVvpr-. Treatment with noninfectious particles produced a ratio of 2.15 HIV*vpr*+/HIV*vpr*-. As this ratio should factor out the effects of other virion associated proteins, we can assume that the change is due to virion associated Vpr protein. Thus, virion associated Vpr is sufficient to induce a similar, though decreased, increase in TNF alpha response to LPS stimulation as that seem in HIV*vpr*+ and HIV*vpr*- infected macrophages. These results indicate that noninfectious particles, present in a significant proportion in the host, may play a role in the immune dysregulation associated with HIV infection.

Contribution of host genetics to Vpr regulation of TNF alpha response to LPS

The results attained thus far indicate that Vpr regulation of the TNF alpha response to LPS differs in each donor tested. The data presented were the result of controlled *in vitro*

experiments, in which the cell type, virus batch, LPS stimulation, and other experimental conditions were held constant. This implicates differences in host genetics as a controlling factor in TNF response. Similar theories have been proposed in the published literature (Kahler, 2005, Sallakci, 2005, D'Alfonso, 1994). While the TNF alpha coding sequence remains highly conserved, specific polymorphisms in the promoter have been investigated in many disease states (reviewed in Allen, 1999, Bayley, 2004). TNF alpha was initially chosen for inclusion in this project due to the potential Vpr-influenced transcription factor sites in its promoter. Thus, the promoter presents the most likely area of effect of host genetic differences. Here, we will determine the role of polymorphism present in the TNF alpha promoter of individual patients, with the goal of determining whether TNF alpha promoter polymorphism presents a source of variation potentially affecting Vpr regulation of the TNF alpha response to LPS.

Determination of TNF promoter polymorphism by DHPLC

To date, investigations of the TNF alpha promoter in disease states have been conducted via case control studies looking at a single promoter polymorphism. The methods previously available, mainly restriction fragment analysis, were not conducive to an investigation of the entire promoter. We chose to use denaturing high performance liquid chromatography (DHPLC) due to its capacity for high throughput analysis of the entire promoter, while remaining highly sensitive to single nucleotide polymorphism (SNPs). DHPLC is a powerful technique for discerning slight changes in the migration of an oligonucleotide due to differential mobility of its different nucleotide base pairs. Thus, for SNP analysis, individual promoters are amplified and then heteroduplexed to a standard individual sample. Comparison to a standard, usually chosen to be a homozygote, allows one to detect sequence variation between samples. Changes in elution through the column caused by mismatched bases between the homozygous standard and the individual sample in question will cause a slight shift in the temperature at which the sample elutes from the column. Thus, polymorphisms will be apparent by populations migrating at different speeds through the column, denoted as splits and shifts in the peak trace. The maximum amplicon which can be used in DHPLC is 700 base pairs. Thus, for this study, the approximately 1300 base pair TNF promoter was divided into three regions. Since the ends of a region are not well resolved due to local changes in the melt temperature at the end of a

fragment, regions are recommended to overlap by at least 50 base pairs. Thus, the TNF promoter region and a portion of the upstream and downstream areas were divided into three regions, each with an approximate 50-60bp overlap. These regions and their amplification parameters are depicted in Materials and Methods, Figure 12.

Initial testing to determine optimal DHPLC conditions was done in a small number of patients (n=5) from whom abundant gDNA was available. Within this limited population, samples were heteroduplexed only to themselves, not to a standard, but dual peaks representing heterozygotes were apparent, indicating the potential for significant polymorphisms to be present. In order to both gain insight into the background variation in the promoter regions present in the population, as well as to find a homozygous individual for use as a standard in screening the macrophage donor samples used in previous experiments, a larger population was screened. Healthy Caucasian donor samples were available from the Pittsburgh Healthy Women Study and 192 samples were screened for polymorphisms. The resulting peaks were then categorized and a distribution was generated by the software. Results are presented in Figure 20. The presence of a single polymorphism within a region will present as three distinct populations. The two homozygous populations, one for each of the two base variations, will separate at opposite ends of the distribution due to the difference in melt temperature introduced in the fragment by substitution of one base. The heterozygous population, with two peaks representing the two species of base variation present, will be included in the middle of the distribution. This type of distribution is seen in Figure 20A for region T1. The results attained for regions T2 and T3, though, did not follow this model distribution. Peak profiles were difficult to sort into populations due to the variability of the peak traces attained. These results indicate that significant variation exists in the TNF alpha promoter, making this an unsuitable study for the use of DHPLC. Based on these, we then assessed the promoter polymorphisms using a more sensitive sequencing analysis.

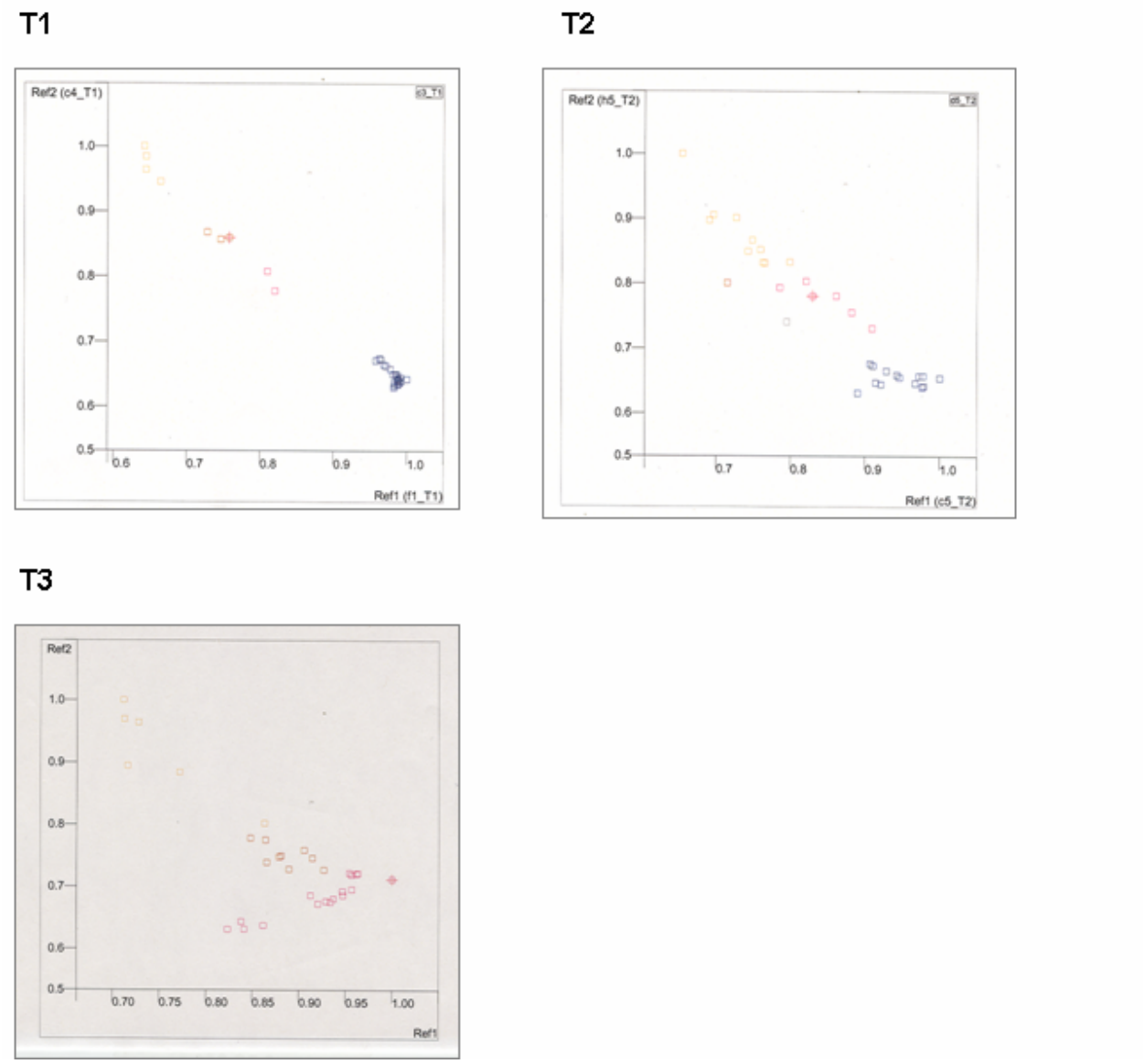


Figure 20: DHPLC analysis of polymorphism in the Caucasian population.

Regions T1, T2, and T3 were amplified as in Materials and Methods and heteroduplexed within themselves. DHPLC was carried out, with the optimal temperatures being 61°C for T1 and T2 and 63°C for T3. Populations were discerned by identifying variance in the peak traces attained and were pseudocolored by the user. Each circle is representative of a group of donors showing a similar peak tracing profile. Axes are assessments of correlation generated by the analysis software.

Analyses of TNF promoter polymorphism by sequencing

The results of the DHPLC indicated that the TNF alpha promoter is highly polymorphic, necessitating a technique capable of accurately identifying several potential SNPs within a region. Sequencing of the entire TNF alpha promoter region was an attractive option, as it provided several advantages. The majority of studies of the TNF alpha promoter to date have been case control studies of single polymorphisms (Bayley, 2005). Thus, little background information is available on the extent of variation of the TNF alpha promoter in normal individuals. Sequencing of the entire promoter region of the macrophage donors will provide data on the amount of variation present in single promoters. This may allow for a more complex analysis, if it is found that patient promoters potentially contain more than one polymorphism. In addition, current techniques, such as fluorescent polarization and SNP analysis by Taqman chemistry only allow for the study of known polymorphisms. Sequencing of the promoter region in this population allows for the potential for new SNPs to be considered.

In order to characterize the promoter of the macrophage donors used in the preceding experiments, genomic DNA extracted from these donors was amplified by PCR. The entire TNF alpha promoter region, termed T-total (Figure 12), was amplified to provide a longer template for the sequencing reaction than the T1-T3 regions used for DHPLC analysis. Longer sequence reads will provide a greater overlap of regions and higher confidence of the consensus sequence attained. All donors were then sequenced using the T1, T2, and T3 forward and reverse primers generated for amplification (Figure 12). The sequencing reaction was carried out at the University of Pittsburgh Genomics and Proteonomics core facility using fluorescent dideoxy nucleotide chemistry. The resulting chromatographs were visually inspected for errors and ambiguous bases (coded as per IUPAC naming, Table 4). Bases were considered ambiguous only when the overlapping forward and reverse reads corresponded, both showing two peaks of equal magnitude for two bases at the position in question. Within each donor, the six resulting sequences were aligned to create a consensus sequence for each donor. Consensus sequences were aligned and deviations from the GenBank #M16441 consensus were mapped. Of note, six deviations from the GenBank sequence were found which were not considered as single

nucleotide polymorphisms (SNP). At each of these six positions, all of the macrophage donors tested showed the same base deviation, indicating a potential "mistake" in the GenBank

Table 4: IUPAC Accepted Base Codes

Letter Designation	Nucleotide
A	Adenine
G	Guanine
U	Uracil
R	A or G
S	C or G
K	G or T/U
H	A, C, or T/U
B	C, G, or T/U
C	Cytosine
T	Thymine
M	A or C
W	A or T/U
Y	C or T/U
V	A, C, or G
D	A, G, or T/U
N	Unknown

consensus sequence. In addition, twenty patient genomic samples attained as part of a corollary study were also sequenced in the TNF alpha promoter region and found to contain the same deviations. Thus, such a "mistake" in the GenBank sequence may have been a true aberration produced via PCR or sequencing or may be due to a low probability highly variant donor used to attain the GenBank sequence. After ruling out the above six deviations, six additional sites were noted to contain deviations from the GenBank sequence. The deviations at these positions occurred with variable frequency, and were manifest as both homozygous and heterozygous. Table 4 indicates the letter abbreviations used to code for sequenced bases. Heterozygous sequences were stringently only considered heterozygous if; A) Both peaks were of equal size and B) Dual peaks were clearly visible on both the forward and reverse sequences. Heterozygous sequences are thus denoted by the letters specified in Table 4: R, S, K, M, W, or

Table A- Ratio of HIVvpr+ versus HIVvpr-

		T	C	C	G	G	G
	wt/vpr- ratio	**-1031**	**-963**	**-957**	**-376**	**-308**	**-238**
FW37649	1.01	T	C	C	G	R	G
KJ74673	1.40	T	C	C	G	G	G
FW40896	1.43	Y	C	C	R	G	R
FV10002	1.67	T	C	Y	G	R	G
FJ00390	1.70	T	C	C	G	G	G
K595253	1.70	Y	C	Y	G	G	R
KJ75874	1.71	Y	C	Y	G	G	R
VA00003	1.74	T	C	C	G	G	G
FW37976	1.77	T	C	C	G	G	G
LV56073	1.88	T	C	C	G	G	G
FW40248	2.05	Y	M	C	G	R	G
VA000021	2.07	T	C	Y	G	G	G
VA00004	2.76	T	C	C	G	G	G
LV56424	2.78	C	A	C	G	G	G
LV57222	3.06	T	C	Y	G	G	G
LV57775	3.24	T	C	C	G	A	G
VA00002	3.50	T	C	C	G	R	G
FW38482	8.50	Y	M	C	G	G	G

Table B- LPS in the absence of virus infection

		T	C	C	G	G	G
Donor ID	**LPS**	**-1031**	**-963**	**-957**	**-376**	**-308**	**-238**
FJ00390	123.36	T	C	C	G	G	G
LV57222	365.70	T	C	Y	G	G	G
FV10002	404.09	T	C	Y	G	R	G
K595253	454.31	Y	C	Y	G	G	R
FW37649	508.64	T	C	C	G	R	G
FW37976	741.67	T	C	C	G	G	G
KJ75874	1752.59	Y	C	Y	G	G	R
VA00004	1890	T	C	C	G	G	G
FW38482	1985.35	Y	M	C	G	G	G
VA000021	2663.79	T	C	Y	G	G	G
LV57775	3112.5	T	C	C	G	A	G
LV56424	3555.56	C	A	C	G	G	G
VA00002	3935.77	T	C	C	G	R	G
FW40248	5236.11	Y	M	C	G	R	G
FW40896	8520.83	Y	C	C	R	G	R
VA00003	15020	T	C	C	G	G	G

Figure 21: Sequence analysis of the TNF promoter.

The TNF promoter T-total region was amplified from all macrophage donors from whom gDNA was available. Regions T1, T2, and T3 sequenced via automated fluorescence dideoxy nucleotide sequencing on an ABI 3730. Six sequences attained from the forward and reverse sequencing of each region were aligned to construct a consensus sequence for each macrophage donor. Bases not denoted below as polymorphic remained 100% conserved throughout the samples tested. A: Sequences listed in order of ascending ration of LPS production from HIVvpr+ versus HIVvpr- infected macrophages stimulated with LPS. B: Sequences listed in order of ascending basal response of donor macrophages to LPS stimulation.

Y. Thus, these positions were considered to contain single nucleotide polymorphisms and are depicted in Figure 21. All bases other than those noted in this graph displayed 100% conservation across all donors tested.

We were then interested in determining whether a correlation existed between the TNF alpha promoter polymorphisms and Vpr mediation of TNF alpha production in response to LPS. Figure 21 is a table summarizing the values for the TNF alpha ELISA and sequence variations for each donor for whom both values were available. In order to ascertain the effects of Vpr, the table is arranged by increasing response to Vpr, increasing TNF alpha production in cells infected with HIV*vpr*+ versus HIV*vpr*- in response to LPS. Precursory visual inspection of the chart suggests that the -238 position shows variation mainly in the "low" group, donors producing a ratio of less than the median of 1.77 ng/ml TNF alpha in HIV*vpr*+ versus HIV*vpr*- infected cells in response to LPS. In the low producing group, the frequency of the heterozygote is 37.5%, while it was 0% in the remaining medium-high producers. The -308 allele showed variation across the maximal range of TNF alpha ELISA ratios, with a heterozygote occurring in the lowest ratio sample as well as one of the highest samples. This suggests that no direct correlation exists between the -308 SNP and Vpr-mediation of TNF alpha production in response to LPS. The -957 and -1031 SNPs show similar results to the -308, with variation throughout the spectrum of TNF alpha ELISA values, suggesting against a direct correlation between these SNPs and Vpr's effects on TNF production. Position -376 only showed variation in one patient, which does not provide sufficient data for determining whether a correlation exists at this SNP position. At the -963 position, variation is found mainly in the medium-high ration group. The medium-high ration donors possessed a variation at this position at a frequency of 33.3%, while there was 0% variation at this position in the low ration donors, implicating a potential correlation between this position and Vpr-mediation of the TNF response to LPS.

The extensive information provided by the sequence analysis allowed us to address another question regarding to TNF alpha production in response to LPS. Studies have suggested that TNF promoter polymorphism may affect the amount of TNF alpha production and have been correlated with diseases caused by TNF overproduction, such as rheumatoid arthritis and malaria (Ubalee, 2001, Bayley, 2004, Flori, 2005). Work in these systems suggests that some hosts may be predisposed to greater TNF alpha production. Thus, it is of interest to determine whether the polymorphism found to be associated with Vpr's effect on TNF alpha production are

also associated with higher basal TNF alpha response to LPS stimulation. This further addresses a question we asked in regards to Figure 16, which was whether those donors who produced a higher TNF alpha HIV*vpr*+/HIV*vpr*- ratio were those who simply had a more robust TNF alpha response to LPS in general. A similar pattern of genomic variation would indicate that TNF is augmenting the basal response. In Figure 21, Table B presents the genomic sequence data in relation to the response of each donor to LPS alone, in the absence of virus infection. Information is sorted into ascending TNF alpha production in response to LPS. Comparison with Figure 21, Table A immediately reveals a difference in the pattern of polymorphisms. The -238 polymorphism is now dispersed across the range of TNF alpha production, with a SNP at this position seen in both one of the highest and one of the lowest TNF alpha producing donors. The polymorphisms seen at positions -308 and -1031 remain dispersed throughout the range. Interestingly, the -963 polymorphisms remains associated with higher TNF alpha production. Position -963 shows a trend towards increased frequency in the low TNF alpha producing donors, although a larger sample size will be necessary to determine if this change is significant. Altogether, there is a clear difference in the patterns of genomic variation correlated with LPS production in response to LPS alone or as a ratio in HIV*vpr*+ versus HIV*vpr*- infected cells. This suggests that Vpr is not augmenting the basal TNF alpha response, but mediating its effects through different promoter elements, perhaps through recruitment or binding of additional cellular transcription factors.

TNF alpha induced apoptosis in bystander T cells

The results attained thus far in this section have characterized a Vpr mediated change in the host cell's production of TNF alpha in response to stimulation. In all donors except one, the presence of Vpr was correlated with an increase in TNF alpha production ranging from 1.39-8.5 fold above the HIV*vpr*- infected sample. We now seek to begin to address the potential effects of the Vpr mediated increase in TNF alpha secretion on the surrounding uninfected cells.

TNF alpha has been shown to mediate not only inflammatory responses, but also to mediate a major pathway contributing to cellular apoptosis. These pathways diverge shortly after TNF binding to its receptor at the cell surface. Downstream effects of TNF alpha binding include caspase 8 and 3 activation and eventual cell death. Thus, while TNF alpha is an

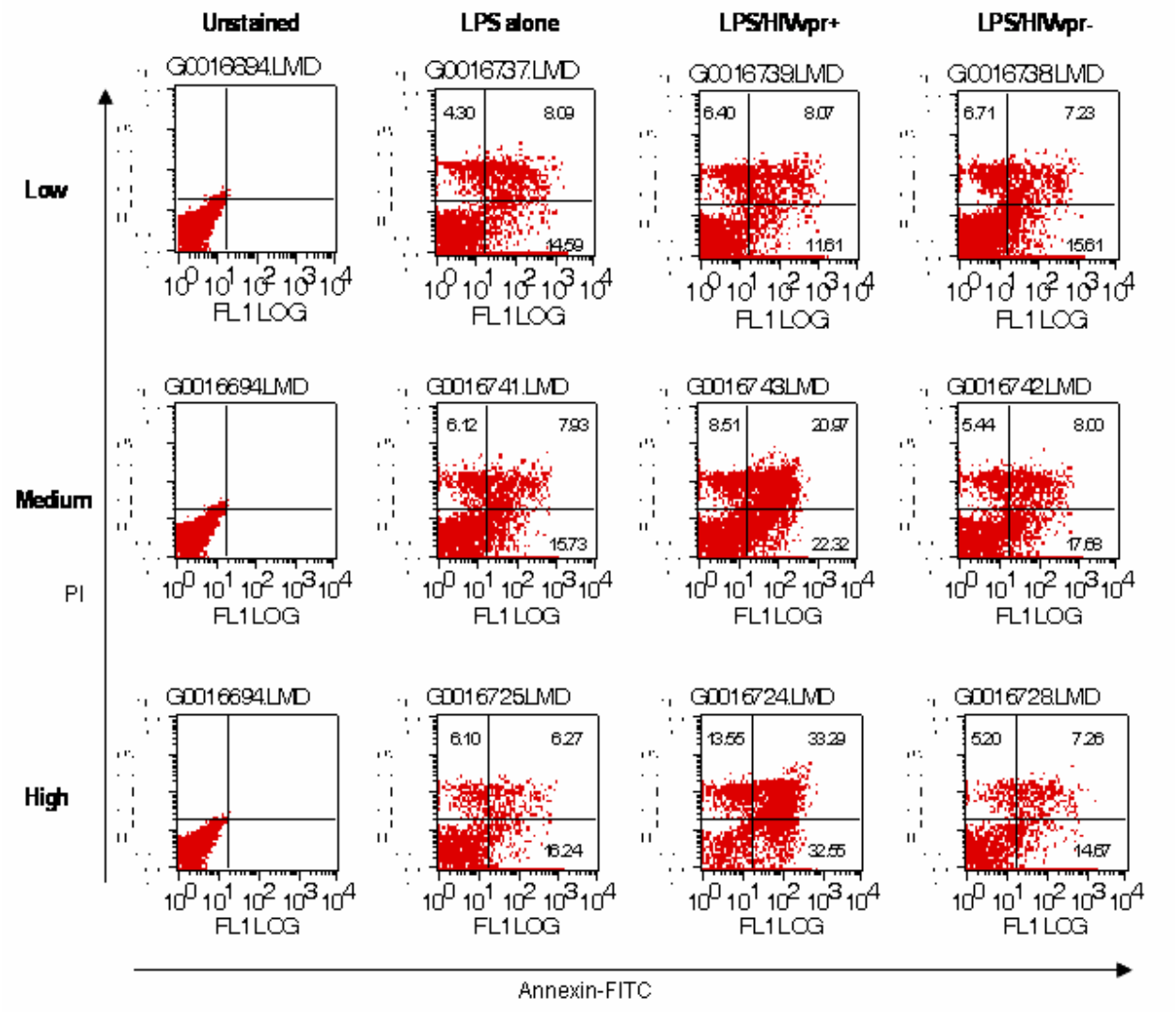


Figure 22: Flow cytometry analysis of apoptosis in bystander cells:

PBLs from an uninfected donor were treated with medium from macrophages producing low (ELISA ratio below median of 1.7), medium (ELISA ratio 1.7-3.0), and high (ELISA ratio >3) TNF in response to LPS stimulation. Medium was centriguged and filters (0.22µm) to remove confounding cell debris prior to use. Apoptosis was assessed by staining with the Annexin-FITC Apoptosis kit (BD Pharmigen). Y axis denotes staining with propidium iodide (PI), X axis denotes staining with Annexin FITC. Lower left quadrant is cells staining negative for both PI and Annexin, live cells. Lower right quadrant is cells staining positive with Annexin only, indicating early apoptosis. Upper right quadrant is cells staining positive with both Annexin and PI, indicating late apoptosis. Apoptotic cells were considered to be the sum of the right quadrants. Results representative of two donors and six supernatants tested.

important mediator of the immune response to pathogens, overproduction of TNF alpha may have deleterious effects, such as initiating apoptosis. To address this, supernatants from donors producing TNF alpha from HIV*vpr*+/HIV*vpr*- infected cells at low (less than the median of 1.77), medium (1.77-3.00) and high (>3.00) levels were used to treat uninfected donor cells. This system mimics the effects of Vpr-mediated changes in TNF alpha secretion from infected macrophages on bystander cells. Supernatants from macrophages uninfected or infected with HIV*vpr*+ or HIV*vpr*-, and stimulated with LPS were sorted into low, medium, and high TNF alpha producers. PBMCs from normal, healthy donors were incubated for 72 hours in low, medium, and high producing supernatants. Induction of apoptosis was assessed by staining with the BD Annexing-FITC apoptosis kit, which utilizes staining with Annexin V and propidium iodide to discern the presence of apoptotic cells in a population. Results presented in Figure 22 indicate a trend of increasing apoptosis induction and cell death with increasing TNF concentration in the supernatant. Propidium iodide staining, indicative of general cell membrane breakdown, is shown on the Y axis. Annexin V staining, specific for changes at the cell membrane associated with apoptosis, is shown on the X axis. Apoptotic cells are considered to be those staining positive for Annexin V. Cells in late stage apoptosis would be expected to begin to experience a breakdown of the cellular membrane, making them permeable to propidium iodide. Thus, percent of apoptotic cells was considered to be the sum of cells staining Annexin positive (lower right quadrant) and cells staining both Annexin and PI positive (upper right quadrant). Percent of cells staining apoptotic appeared to increase in correlation from the low to medium and high supernatant treated cells. This suggests a correlation between Vpr-mediated increase in TNF alpha levels and bystander cell death, although the effects of other apoptosis inducing molecules in the supernatant cannot be ruled out. Macrophages have also been shown to produce soluble Fas ligand in response to stimulation. Soluble Fas ligand (FasL) induced cell death through receptor mediated signaling in a manner similar to TNF alpha. The levels of FasL in the supernatants were assessed by ELISA and were found not to correlate with increased TNF alpha levels, ruling out a confounding effect of this molecule on the apoptosis seen with increasing Vpr-mediated TNF alpha production. These experiments suggest that TNF alpha may be one of the secreted factor(s) which have been suggested as explanation for bystander cell death caused by HIV-1 Vpr. The results of this *in vitro* analysis represent an

important proof of concept test for TNF alpha as the secreted factor which has been alluded to, this remains to be further tested in the more complex milieu of the immune cell in vivo.

4.4 DISCUSSION

Recent work in our laboratory and others has suggested a role for Vpr in modulation of host immune function (Majumder, 2005). The described effects of Vpr have been extensive for a viral protein, thus several studies, including ours have implicated the potential for induction of a secreted factor, such as a cytokine or chemokine in mediating some of the indirect effects of Vpr. Results attained in Chapter 2 of this study suggested that TNF alpha promoter possess motifs/cis elements capable of being modulated by Vpr. The TNF alpha promoter contains transcription factor binding sites similar to those through which Vpr acts on the HIV-1 LTR to modulate transcription. Secreted TNF alpha is highly active in the immune system, capable of inducing both a pro-inflammatory immune response and apoptosis. It was therefore of interest to determine whether Vpr, in the context of virus infection, was capable of modulating TNF alpha production from infected cells. As one of the subsets of cells thought to be infected by virus crossing the mucosal membrane, macrophages play an important role in HIV pathogenesis (Smith, 2001, Lin, 2002). In addition, macrophages are capable of producing high amounts of TNF alpha when stimulated and are important in immune response to pathogens. Thus, here, we chose macrophages to study, being one of the host cells most likely to be of importance in vivo if Vpr was found to affect TNF alpha secretion.

When I first began this experiment, the intent was to infect four donor macrophages with HIV*vpr*+ and HIV*vpr*- virus, follow that with stimulation, and determine the level of TNF alpha production. I assumed that four repeated experiments would produce an acceptable mean and provide a clear cut answer to whether Vpr was capable of affecting TNF alpha secretion, manifest as an up- or down-regulated ration in HIV*vpr*+ versus HIV*vpr*- infected cells. After four experiments, two things became apparent. First, Vpr modulated TNF alpha expression in both LPS and CD40 ligand stimulated cells, indicating an effect independent of stimulation pathways. Second, that Vpr modulation of TNF alpha varied greatly between host donor

macrophages used. Each of the four experiments conducted produced a different ratio of TNF alpha secretion from HIV*vpr*+ versus HIV*vpr*- cells. In fact, TNF alpha secretion from uninfected macrophages stimulated with either LPS or CD40 Ligand also differed between hosts. This finding was corroborated by a literature search, which showed that in diseases such as malaria, patients with exacerbated pathogenesis produced higher levels of TNF alpha in response to the same pathogen (Ubalee, 2001). In light of this information, we undertook a larger scale study in an attempt to define the upper and lower limits of Vpr mediation of TNF alpha secretion.

Twenty-two normal healthy donor sets of macrophages were isolated, matured, infected with HIV*vpr*+, HIV*vpr*-, or left uninfected, and stimulated with LPS. Due to the cell numbers needed within each donor to allow for all three variations of treatment, as well as repeated in duplicate within donors, stimulation with CD40L was not continued after results proved to be similar in the first six experiments (original four plus two additional). Additionally, unstimulated macrophages, both infected and uninfected, continued to show levels of TNF alpha which were low to undetectable by ELISA, thus these samples were excluded from further studies. As a host effect on TNF alpha secretion was implicated, we attempted to control as many factors as possible in order to make host donor cells the only major variable. To this effect, all experiments were undertaken using the same batches of virus and batch and lot of LPS, which were frozen as aliquots. Results showed a range of TNF alpha secretion levels from HIVvpr+ versus HIVvpr- infected cells from a ratio of 1.01 to 8.5 (Figure 13). While the level of upregulation varied around a median of 1.77, no donor was found to experience a decrease in TNF alpha secretion in the presence of Vpr. This is in agreement with the promoter map attained in Aim 1, which showed binding sites for Sp1, NF-kB, and GR, all transcription factors through which Vpr induced increased transcription.

Due to the known effects of steroid hormones on both TNF alpha secretion and HIV pathogenesis, we investigated whether the variant TNF alpha production seen correlated with sex of the donor. The sample used was composed of a nearly equal number of male and female donors, and no significant difference was seen between the groups, with the means and medians being almost identical (Figure 15). Though significant differences have been found between male and female in TNF alpha production as well as a hormonal effect on HIV pathogenesis, no significant correlation was seen in here. In addition, we were interested in whether the donors

producing high levels of TNF alpha in the presence of Vpr were those donors producing the highest levels of TNF alpha in response to LPS stimulation in general. TNF alpha secretion ranged from 123 pg/ml to 15020 pg/ml, indicating that a greater than 100 fold difference can be seen in patient response to LPS. Yet, when raw TNF alpha ELISA values in response to LPS stimulation were overlayed with the HIVvpr+/HIVvpr- ratios representing Vpr mediation of TNF alpha, no correlation was seen (Figure 16). The highest outlier, the donor producing an 8.5 ratio, was also removed in order to expand the graph, but the correlation coefficient (R^2) remained below 0.01, indicating no correlation was present. Thus, the donors responding most readily to Vpr presence are not necessarily the donors predisposed to higher levels of TNF production in response to stimulation in general. This result is interesting because it suggests that there is a different or additional host component responsible for Vpr mediation of TNF alpha levels in HIV infected cells in response to stimulation than that controlling the basal TNF alpha levels in response to stimulation.

Before undertaking a further investigation of host factors involved in TNF alpha secretion, we first sought to determine whether the effect of Vpr on TNF alpha secretion is mediated at the transcriptional level. Results attained in Aim 1 (Chapter 2) of this study suggest that Vpr exerts significant effects on the transcriptional level of host cellular genes. In addition, the predicted promoter similarities to the LTR, suggest the potential for Vpr effects on transcription factor binding and thus mRNA production. This question was addressed using sensitive real time RT-PCR and Taqman chemistry, allowing for high sensitivity and specificity in mRNA level determination. To maintain consistency in this study, I first attempted to measure TNF alpha mRNA in the same cells utilized for ELISA results, which had been collected, flash frozen in liquid nitrogen, and stored at -80°C after 24 hour LPS stimulation. While TNF alpha mRNA was detectable in these samples, the ratios in HIV*vpr*+ versus HIV*vpr*- infected cells were not comparable to those seen with the protein. Upon search of the literature, I found two papers showing that TNF alpha mRNA transcription can occur as early as one hour post stimulation by LPS. Thus, new donor macrophages were attained and a time course experiment was undertaken. Due to differences in the two reports cited, I was unsure exactly at what time point maximal TNF mRNA would be detectable, two slightly different timecourses were planned in the two donors used. In both donors, we found a significant increase in TNF alpha mRNA levels in HIV*vpr*+ infected cells stimulated with LPS versus HIV*vpr*- infected cells

stimulated with LPS or LPS alone treated cells. TNF alpha mRNA was highest at 4-6 hours post LPS stimulation, but was donor dependent. This suggests that donors may respond to stimulation on a slightly different time scale, providing an explanation for the differences in time post stimulation to TNF alpha mRNA production in vitro reported in the literature.

The effects of Vpr on TNF alpha mRNA transcription combined with the predicted similarities between the TNF alpha and LTR promoters, lead us to investigate the TNF alpha promoter as a source of donor variability in TNF alpha response to LPS stimulation. When I began this study, it seemed inconceivable that much of the work on the TNF promoter had not already been done. After exhaustive literature searches, I found that most of the information available on the TNF promoter was incomplete and often inconsistent. The self-described weakness of most studies is that they were conducted as patient based case-control studies (Bayley, 2004). The complexities of the regulation of TNF alpha, controlled by not only transcriptional regulation, but by several other cytokines and at the translational level, makes the study of this promoter in patients difficult. Thus, information regarding the effects the TNF alpha promoter on disease remain inconsistent. What is consistent is agreement that the promoter of TNF alpha contains polymorphisms which have been predicted to affect disease states. The most conclusive results have been attained in the study of rheumatoid arthritis and malaria, both diseases in which inflammatory responses are an important mediator of the pathogenesis of the disease. In this study, we sought to determine whether TNF alpha promoter polymorphisms existed which may correlate with Vpr-mediated increase in the TNF alpha response to LPS. The *in vitro* controlled system utilized for the ELISA experiments provides and excellent system in which to study TNF alpha regulation, devoid of many of the confounding factors present in patient populations.

Genomic DNA collected from the macrophage donor patients utilized in the preceding experiments was extracted and subjected to genetic analysis. Initial studies intended to make use of the DHPLC analysis to assess and categorize promoter polymorphisms. The TNF promoter proved to contain too much variation for DHPLC to be useful. The presence of more than two distinct variations in a region subjected to DHPLC analysis make resolution of the peaks difficult and analysis faulty. Sequencing analysis was then chosen to complete the study of the TNF promoter in the macrophage donor samples. Sequencing carried the disadvantage of being a more labor intensive and low throughput system, thus we were not able to study background

variation in the Caucasian population in large numbers of healthy donors, as originally intended. An advantage of sequencing was that it was able to provide a significant amount of data for the experimental macrophage donors. Analysis of the entire promoter revealed the presence of six polymorphisms, present at varying frequencies within the samples tested. SNPs at positions -238 and -963 appear to correlate with Vpr induced mediation of the TNF alpha response to LPS. This result is interesting given the reported correlation between the -238 TNF alpha promoter polymorphism and HIV associated lipodystrophy (Maher, 2002). HIV-associated lipodystrophy is a syndrome characterized by abnormal fat distribution affecting mainly HIV patients on highly active retroviral therapy regimens. Vpr has also been implicated in exacerbation of HIV associated lipodystrophy through its effects on the steroid receptor family member, the glucocorticoid receptor (Kino, 2004, Mirani 2002). A link between -238 TNF alpha promoter SNP and Vpr may present a potential mechanism for Vpr's involvement in lipodystrophy. To this end, our laboratory has received 20 samples from HIV+ patients with and without lipodystrophy. Genetic analysis of these samples has revealed polymorphisms at the same six sites found in this analysis. Further analysis of the role of Vpr in TNF promoter polymorphism may provide insights into the mechanism of lipodystrophy and identify predisposing factors. As lipodystrophy and its hallmark symptoms, including a "buffalo hump" fat deposit on the upper back presents a significant source of nonadherence to HIV medications, such research may benefit patient quality of life (reviewed in Corless, 2005).

The full sequencing analysis carried out here also allowed us to address another question regarding Vpr's effects on TNF regulation. Variation in TNF levels in response to LPS stimulation varied across donor samples by a magnitude of greater than 100. Thus, individual variations in susceptibility to TNF induction would explain the variable effects seen for HIV Vpr. While this reasoning presented a logical explanation for variability, further analysis suggested a more complicated mechanism. When polymorphism data was arranged according to increasing TNF production in response to LPS, a different pattern emerged from that seen in the Vpr data. The -238 polymorphism no longer clearly associated with TNF levels, while the -957 polymorphism now did. This data indicated that in mediating its effects on the TNF promoter, Vpr may utilize a different region and thus different transcription factors. This data is supported by results attained by ELISA, which showed that when TNF levels were overlaid with HIVvpr+/HIVvpr- ratios, no correlation was found. The -238 position is involved in a motif

combining triplicate binding sites for the transcription factor Sp1 and a p300 transcription factor binding site. Vpr is known to bind to Sp1 and enhance transcription from the HIV- LTR through a triplicate Sp1 site proximal to the start of transcription. In addition, Vpr has been found through pull down experiments to be associated p300, although it is still questionable whether this is a direct binding or whether the association is through a larger transcriptional complex binding sites. Further studies could utilize EMSA (electrophoretic mobility shift assay) and CHIP (chromatin immunoprecipitation) to determine the effects of Vpr on binding of these transcription factors to the TNF promoter. Regulation of TNF alpha by transcription factor binding has been postulated to be cell type specific, thus the studies presented here have been expanded to include cell lines susceptible to HIV infection and TNF induction, beginning with dendritic cells.

The results attained here made use of a controlled *in vitro* system to identify changes in TNF alpha secretion. A study of HIV patient levels of TNF alpha mRNA also showed an increased level of TNF alpha protein and mRNA in HIV patients versus healthy controls. Yet, given the results attained in this study, I would question the validity of cross donor studies. Differing TNF alpha levels on an order of magnitude of greater than 100 may confound results attained across donors in vivo. Results presented here will provide the basis for further molecular analysis of the effects of Vpr on TNF promoter regulation. In addition, studies undertaken in HIV positive patients will be important in determining whether the results presented here, implicating the effect of Vpr on the TNF promoter as the missing link between Vpr and HIV associated lipodystrophy will prove true. Genetic predisposition to this syndrome may prove important in designing HARRT regimens with the lowest possible side effects, generating higher adherence and quality of life for HIV patients.

5.0 IMPLICATIONS AND FUTURE DIRECTIONS

The results of the study presented here will be expected to add to the growing knowledge of the molecular functioning of HIV within the host cell. Specifically, these results forward the study of the accessory gene Vpr. The underlying hypothesis that Vpr causes differential regulation of host cellular genes was based on studies in ours and other laboratories which had shown that Vpr bound cellular transcription factors and was able to promote transactivation of promoter reporter constructs (reviewed in Section 1.2.9). These studies, though, were conducted in transfected cell culture studies in which constructs were overexpressed and Vpr expressed in the absence of other viral proteins. Thus, the question remained whether these transactivation properties would extend to cellular promoters and genes in the more complicated environment of viral infection. During HIV infection, co-expressed viral proteins may cooperate to produce effects different from those for each protein expressed alone. Alternately, the stepwise processes of infection and replication may focus cellular resources differently than that seen in transfection experiments. Thus, in order to study the effects of Vpr on cellular transcription in the context of virus replication, we utilized isogenic virus expressing or not expressing Vpr. The background of infection was identical in both the control and test sample, the only factor being Vpr expression. In addition, primary human lymphocytes were utilized as opposed to cell lines in order to more closely mimic the cellular environment HIV is exposed to *in vivo*. In fact, the extent of regulation was surprising. More than 15 distinct classes of cellular genes were differentially regulated by Vpr on the microarray and antibody array analyses combined. This amounted to more than eighty-nine total genes being regulated at a level of two-fold or greater (Tables 1, 2, and 3), roughly 2% of the greater than 4500 genes tested. Two percent of the host gene profile is striking as an extensive change to be induced by a small, exogenous protein. This

prompted me to theorize that Vpr must be targeting shared motifs in the differentially regulated genes.

Extensive search of the promoter regions of several representative genes regulated by Vpr indicated that genes differentially regulated by Vpr may share common transcription factor binding sites, dependent on whether they were up or downregulated. Specifically, genes upregulated by Vpr appeared to share GR and Sp1 binding sites, while downregulated genes possessed Oct-1 binding sites proximal to the start of transcription (Section 2.6.2, Figure 5). The results of this analysis, though, were limited by the fairly low predictive power of transcription factor binding site prediction software, as well as the labor intensive nature of promoter mapping (Pederson, 1999). Transcription factor binding sites tend to be short, degenerative sequences, making them difficult to predict with a high degree of accuracy (Hu, 2005, Qiu, 2003). A more powerful and practical approach to high-throughput analysis which has recently been introduced is pathway analysis. This analysis technique utilizes analytical software which curates the pathway/cell signaling data in available databases. Results from high-throughput analyses are entered into this software and a resulting map of potential pathways linking the regulated genes is generated. The placement of differentially regulated genes in known pathways is also shown, allowing one to extrapolate other genes which may be regulated, thus generating testable hypotheses on changes in cellular signaling from array data. Two pathway analysis programs which have begun to appear in peer-reviewed literature are Ingenuity (www.ingenuity.com) and PathwayAssist (http://www.ariadnegenomics.com). Presently, these novel programs remain highly expensive. Re-analysis of the data presented here may be prudent when these programs become more widely available. Such analyses may provide new leads, as well as confirm the pathways implicated in Chapters 3 and 4.

Even in the absence of array analyses, we were able to group the genes and look for common themes of regulation. One common theme of regulation which emerged was that of immunoregulatory molecules. A previous study by Ayyavoo, et. al. (1997) indicated that Vpr may have immunosuppressive properties. The downregulation of five immune molecules in this study (Table 1), lead us to question whether Vpr may be regulating more immune molecules than those included on the relatively small microarray chips used here. Thus, Vpr-mediated transcriptional regulation was added to another already ongoing study in our laboratory on Vpr immunosuppressive effects in monocytes. As part of that study, I found that in addition to those

listed, Vpr caused downregulation of CD80, CD83, and CD86 at the transcriptional level (Majumder, 2005). Vpr also appeared to upregulate TNF alpha in similar monocyte studies. After confirming the TNF alpha regulation by multiplex Luminex assay, I chose to include TNF alpha in further studies presented here, including the promoter analysis and function studies. Though TNF alpha was not identified on the initial microarray screen, it emerged as a logical outgrowth of those studies and therefore required further analysis in conjunction with these studies. The other gene studied in relation to function was NHE1, which emerged directly from the antibody array analyses. Literature search showed that this protein played an important role in cell homeostasis as well as mediated two apoptotic pathways. Thus, NHE1 was also chosen for inclusion in the promoter analysis (Section 2.6.2) and functional analyses (Chapter 3).

NHE1 was downregulated by a ratio of 0.43 on the antibody array analyses (Table 3). This finding was confirmed by western blot (Figure 6) and downregulation was found to be at the transcriptional level (Figure 7). Interestingly, Vpr-mediated transcriptional downregulation of NHE1 was inhibited by mifepristone, an antagonist of the glucocorticoid receptor (Figure 8). These results suggest that Vpr may be mediating NHE1 downregulation through binding to and acting upon the GR. As described in the Introduction (Section 1.2.9), Vpr has previously been shown to bind specifically to GR and affect transcriptional regulation through this complex. The promoter of NHE1 was shown by in silico analysis as well as in a previous study (Facanha, 2000) to contain glucocorticoid response elements (GRE). Thus, the regulation of NHE1 appeared to be specific and relevant to the previously described functions of Vpr, making it an interesting topic for further study. Literature search indicated that NHE1 had two roles in cellular apoptosis. The first was through its well-described function as an ion exchanger at the cell surface. The capacity of NHE1 to export H+ ions generated during metabolic processes makes it a key factor in maintaining the basal intracellular pH. I therefore hypothesized that a decrease in NHE1 at the cell surface due to NHE1-downregulation may negatively affect the ability of the cell to maintain basal pH. Intracellular pH, as assessed by flow cytometry using was decreased in cells infected with HIV*vpr*+ versus HIV*vpr*- virus (Figure 9). Such a decrease may represent a reduced capacity of the infected cell to dispose of H+ ions generated during metabolism. In addition, HIV*vpr*+ has been shown to replicate more rapidly than HIV*vpr*-. Viral replication requires increased synthesis of viral proteins, intracellular transport, energy expenditure, and thus an increased rate of cellular metabolism would be necessary to

accommodate these processes. Increased production of H+, combined with approximately one half of the NHE1 normally available for transport at the cell surface, would be expected to results in a large decrease in intracellular pH, as seen here. There is one other literature report of viral infection affecting NHE1 activity, though in that report HCMV induces the opposite effect, more alkaline cellular environment (pHi +01.-0.2) associated decreased cytoplasmic buffering (Crowe, 1997). Further study may be interesting in order to determine if other human viruses have evolved the capacity to manipulate the internal cell environment through the NHE1.

A recent paper by Wu et. al. (2004) described another role of NHE1 in cell survival, that of a scaffold at the plasma membrane. NHE1 appears to anchor the ERM complex at the plasma membrane, resulting in increased phosphorylation of the pro-survival Akt kinase. I hypothesized that a decrease in NHE1 at the cell surface due to Vpr-mediated downregulation may result in a decrease in phosphorylated Akt. Western blot analyses of primary macrophages and lymphocytes indicated that this was the case (Figure 10). Active Akt inhibits the pro-apoptotic protein Bad, thus protecting the cell from initiation of apoptosis by Bad at the mitochondria (reviewed in Datta, 1999). While downregulation of a pro-survival pathway appears counterproductive to viral propagation, downregulation of Akt activation is also associated with immunosuppression. Measles virus induces a decrease in activated Akt, resulting in decreased T cell responsiveness and immunosuppression (Avota, 2001). A recent study has also linked the localization and scaffolding of the ERM complex to T cell receptor signaling and activation (Charrin, 2006). Loss of ERM membrane association and Akt activation due to Vpr-mediated NHE1 downregulation uncouples intracellular signaling and may thus facilitate HIV escape from immune surveillance. Although prolonged NHE1 downregulation would eventually lead to apoptosis, this would not be before the cell had served as a protected incubator for virus propagation. Further studies of the localization of the ERM complex in HIV*vpr*- and HIV*vpr*+ cells may prove interesting and provide additional evidence of this immune uncoupling. As multiple cell signaling pathways converge on Akt, studies of the downstream effects of decreased Akt phosphorylation may provide interesting insight into the signaling mechanisms altered during HIV infection.

In order to further protect itself from immune surveillance, HIV induces cell death in uninfected lymphocytes, resulting in the loss of CD4+ lymphocytes defining AIDS. This death is though to be due to release of either viral protein(s) as toxins or through the release of cellular

factors from infected cells, either of which may cause apoptosis. Here, we show that Vpr induced cell death in bystander lymphocytes through its upregulation of TNF alpha in macrophages. Vpr was shown to upregulate TNF alpha at both the protein and mRNA level in primary macrophages (Figure 13). Similar results have been shown for Tat, and further investigations into whether Tat and Vpr symbiotically increase TNF alpha would be interesting. As reviewed in Section 1.9, Tat and Vpr have been shown to function cooperatively in inducing transcriptional activation of the LTR. If LPS-induced TNF alpha upregulation by Vpr is occurring due to the similarity of its promoter to the LTR, as hypothesized in this study, then Tat should and Vpr would be expected to be working cooperatively on the cellular TNF alpha promoter as well.

One of the most interesting results to come from this study came from the observation that the level of induction of TNF alpha in response to LPS by Vpr differed between donor cells used. The presence of Vpr was found to increase the production of TNF alpha after stimulation with LPS by a ratio of 1.0 to 8.5 (Figure 13). Like most other biologic parameters, it would not be surprising to find different levels of TNF alpha between individuals. Yet, in the *in vitro* system used here, the confounding endocrine factors in the body would be removed and we expected the cells to respond uniformly to Vpr. The large range of responses attained above argued that this was not the case. Literature search indicated that promoter polymorphisms were implicated in the regulation of TNF alpha. Sequencing of the promoters of each of the donors revealed the presence of six polymorphisms. Two of these polymorphisms, at positions -238 and -963, appeared to correlate with the ratio of Vpr induction of TNF alpha response (Figure 21). Unfortunately, the small number of sequenced samples in this study is not sufficient to draw statistically significant conclusions regarding these correlations. Yet, this data for the first time implies that Vpr may directly affect the transcription of TNF alpha in the cell. Vpr has been speculated to play a role in HIV dementia (van de Bovenkamp, 2002, Pomerantz, 2004, Burdo, 2004) as well as HIV lipodystrophy (Kino, 2004). The regulation of TNF alpha by Vpr provides a potential link to both of these syndromes. TNF alpha is a potent neurotoxin (Sriram, 2002) and regulates adipose metabolism (reviewed in Hauner, 2005). TNF alpha has also been implicated in both HAD (Huang, 2005) and AIDS-associated lipodystrophy (Haugaard, 2006). Based upon the results attained in this study, the TNF alpha promoters of lipodystrophy patients and HAD patients should be sequenced for polymorphisms. Of interest will also be the sequencing of the

Vpr molecules in each patient. A more complex interaction may occur between the host genetic factors, the Vpr sequence, and potentially the Tat sequence (due to the Vpr-Tat synergism hypothesized above). Elucidation of the interaction between Vpr and TNF alpha may provide a therapeutic target for the treatment of both HAD and lipodystrophy.

The study in this manuscript presents a detailed study of the transcriptional effects of the HIV-1 Viral protein R on cellular gene transcription. The results of the array analyses are the first reports of global cellular gene regulation by Vpr. More importantly, the studies which grew out of the array analyses, those of TNF alpha and NHE1, now present potential mechanisms with roles in Vpr-mediated apoptosis. Further study of these pathways may prove interesting in both treating the lymphocyte death aociated with HIV disease progression, as well as the confounding conditions of HAD and lipodystrophy. Therefore, we hope that the results presented here may aid in the fight against the global epidemic of AIDS.

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ABI PRISM 7700 Sequence Detection User Manual: User Bulletin #2, Applied Biosystems, Foster City, California (1997).

Agnew LL, Kelly M, Howard J, Jeganathan S, Batterham M, Ffrench RA, Gold J, Watson K. R. (2003). Altered lymphocyte heat shock protein 70 expression in patients with HIV disease. AIDS. 17(13):1985-8.

Agostini I, Navarro JM, Rey F, Bouhamdan M, Spire B, Vigne R, Sire J. (1996). The human immunodeficiency virus type 1 Vpr transactivator: cooperation with promoter-bound activator domains and binding to TFIIB. J Mol Biol. 261(5):599-606.

Ahmad N, Venkatesan S. (1988). Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science. 241(4872):1481-5.

Alimonti JB, Ball TB, Fowke KR. (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. J Gen Virol. 84(Pt 7):1649-61.

Allen RD. (1999). Polymorphism of the human TNF-alpha promoter--random variation or functional diversity? Mol Immunol. 36(15-16):1017-27.

Amini S, Saunders M, Kelley K, Khalili K, Sawaya BE. (2004). Interplay between HIV-1 Vpr and Sp1 modulates p21(WAF1) gene expression in human astrocytes. J Biol Chem. 279(44):46046-56.

Andersen JL, Zimmerman ES, DeHart JL, Murala S, Ardon O, Blackett J, Chen J, Planelles V. (2005). ATR and GADD45alpha mediate HIV-1 Vpr-induced apoptosis. Cell Death Differ. 12(4):326-34.

Apolloni A, Hooker CW, Mak J, Harrich D. (2003). Human immunodeficiency virus type 1 protease regulation of tat activity is essential for efficient reverse transcription and replication. J Virol. 77(18):9912-21.

Aquaro S, Bagnarelli P, Guenci T, De Luca A, Clementi M, Balestra E, Calio R, Perno CF. (2002). Long-term survival and virus production in human primary macrophages infected by human immunodeficiency virus. J Med Virol. 68(4):479-88.

Arunagiri C, Macreadie I, Hewish D, Azad A. (1997). A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4+ lymphocytes. Apoptosis. 2(1):69-76.

AVERT.org, HIV and AIDS Statistics, AVERT, http://www.avert.org/statindx.htm

Avota E, Avots A, Niewiesk S, Kane LP, Bommhardt U, ter Meulen V, Schneider-Schaulies S. (2001). Disruption of Akt kinase activation is important for immunosuppression induced by measles virus. Nat Med. 2001 Jun;7(6):725-31.

Ayyavoo V, Mahboubi A, Mahalingam S, Ramalingam R, Kudchodkar S, Williams WV, Green DR, Weiner DB. (1997). HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. Nat Med. 3(10):1117-23.

Ayyavoo V, Muthumani K, Kudchodkar S, Zhang D, Ramanathan P, Dayes NS, Kim JJ, Sin JI, Montaner LJ, Weiner DB. (2002). HIV-1 viral protein R compromises cellular immune function in vivo. Int Immunology.

Azad AA. (2000). Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? Biochem Biophys Res Commun. 267(3):677-85.

Baldwin RL, Stolowitz ML, Hood L, Wisnieski BJ. (1996). Structural changes of tumor necrosis factor alpha associated with membrane insertion and channel formation. Proc Natl Acad Sci U S A. 93(3):1021-6.

Balliet JW, Kolson DL, Eiger G, Kim FM, McGann KA, Srinivasan A, Collman R. (1994). Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate. Virology. 200(2):623-31.

Balter M. (1996). A second coreceptor for HIV in early stages of infection. Science. 272(5269):1740.

Barnhart BC, Peter ME. (2003). The TNF receptor 1: a split personality complex. Cell. 114(2):148-50.

Barnor JS, Miyano-Kurosaki N, Takaku H, Yamaguchi K, Sakamoto A, Ishikawa K, Yamamoto N, Osei-Kwasi M, Ofori-Adjei D. (2005). The middle to 3' end of the HIV-1 vif gene sequence is important for vif biological activity and could be used for antisense oligonucleotide targets. Nucleosides Nucleotides Nucleic Acids. 24(10-12):1745-61.

Barré-Sinoussi1 F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L. (1983). Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). Science. 220, 868-871

Baumgartner M, Patel H, Barber DL. (2004). Na(+)/H(+) exchanger NHE1 as plasma membrane scaffold in the assembly of signaling complexes. Am J Physiol Cell Physiol. 287(4):C844-50.

Bayley JP, Ottenhoff TH, Verweij CL. (2004). Is there a future for TNF promoter polymorphisms? Genes Immun. 5(5):315-29.

Bebenek K, Abbotts J, Roberts JD, Wilson SH, Kunkel TA. (1989). Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. J Biol Chem. 264(28):16948-56.

Bell I, Schaefer TM, Trible RP, Amedee A, Reinhart TA. (2001). Down-modulation of the costimulatory molecule, CD28, is a conserved activity of multiple SIV Nefs and is dependent on histidine 196 of Nef. Virology. 283(1):148-58.

Belshan M, Mahnke LA, Ratner L. (2005). Conserved amino acids of the human immunodeficiency virus type 2 Vpx nuclear localization signal are critical for nuclear targeting of the viral preintegration complex in non-dividing cells. Virology. 2005 Nov 29; [Epub ahead of print]

Berglez JM, Castelli LA, Sankovich SA, Smith SC, Curtain CC, Macreadie IG. (1999). Residues within the HFRIGC sequence of HIV-1 vpr involved in growth arrest activities. Biochem Biophys Res Commun. 264(1):287-90.

Berthoux L, Sebastian S, Sokolskaja E, Luban J. (2005). Cyclophilin A is required for TRIM5{alpha}-mediated resistance to HIV-1 in Old World monkey cells. Proc Natl Acad Sci U S A. 102(41):14849-53.

Besson P, Fernandez-Rachubinski F, Yang W, Fliegel L. (1998). Regulation of Na+/H+ exchanger gene expression: mitogenic stimulation increases NHE1 promoter activity. Am J Physiol. 274(3 Pt 1):C831-9.

Blagoveshchenskaya AD, Thomas L, Feliciangeli SF, Hung CH, Thomas G. (2002). HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. Cell. 111(6):853-66.

Bond J, Varley J. (2005). Use of flow cytometry and SNARF to calibrate and measure intracellular pH in NS0 cells. Cytometry A. 64(1):43-50.

Borman AM, Quillent C, Charneau P, Dauguet C, Clavel F. (1995). Human immunodeficiency virus type 1 Vif- mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. J Virol. 69(4):2058-67.

Bosetti F, Seemann R, Bell JM, Zahorchak R, Friedman E, Rapoport SI, Manickam P. (2002). Analysis of gene expression with cDNA microarrays in rat brain after 7 and 42 days of oral lithium administration. Brain Res Bull. 57(2):205-9.

Bouhamdan M, Benichou S, Rey F, Navarro JM, Agostini I, Spire B, Camonis J, Slupphaug G, Vigne R, Benarous R, Sire J. (1996). Human immunodeficiency virus type 1 Vpr protein binds to the uracil DNA glycosylase DNA repair enzyme. J Virol. 70(2):697-704.

Bour S, Schubert U, Strebel K. (1995). The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. J Virol. 69(3):1510-20.

Bouzar AB, Villet S, Morin T, Rea A, Genestier L, Guiguen F, Garnier C, Mornex JF, Narayan O, Chebloune Y. (2004). Simian immunodeficiency virus Vpr/Vpx proteins kill bystander noninfected CD4+ T-lymphocytes by induction of apoptosis. Virology. 326(1):47-56.

Brin E, Yi J, Skalka AM, Leis J. (2000). Modeling the late steps in HIV-1 retroviral integrase catalyzed DNA integration. J Biol Chem. 275(50):39287-95.

Bruns K, Fossen T, Wray V, Henklein P, Tessmer U, Schubert U. (2003). Structural characterization of the HIV-1 Vpr N terminus: evidence of cis/trans-proline isomerism. J Biol Chem. 278(44):43188-201.

Buckman JS, Bosche WJ, Gorelick RJ. (2003). Human immunodeficiency virus type 1 nucleocapsid Zn(2+) fingers are required for efficient reverse transcription, initial integration processes, and protection of newly synthesized viral DNA. J Virol. 77(2):1469-80.

Bukovsky AA, Dorfman T, Weimann A, Gottlinger HG. (1997). Nef association with human immunodeficiency virus type 1 virions and cleavage by the viral protease. J Virol. 71(2):1013-8.

Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, Haggerty S, Stevenson M. (1992). Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci 89(14):6580-4.

Bukrinsky MI, Sharova N, McDonald TL, Pushkarskaya T, Tarpley WG, Stevenson M. (1993). Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc Natl Acad Sci U S A. 90(13):6125-9.

Bukrinsky M. (2004). A hard way to the nucleus. Mol Med. 10:1-5.

Burdo TH, Nonnemacher M, Irish BP, Choi CH, Krebs FC, Gartner S, Wigdahl B. (2004). High-affinity interaction between HIV-1 Vpr and specific sequences that span the C/EBP and adjacent NF-kappaB sites within the HIV-1 LTR correlate with HIV-1-associated dementia. DNA Cell Biol. 23(4):261-9.

Carr A, Samaras K, Burton S, Law M, Freund J, Chisholm DJ, Cooper DA. (1998). A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. AIDS. 12(7):F51-8.

CDC- Divisions of HIV/AIDS Prevention HIV/AIDS Surveillance Report. http://www.cdc.gov/hiv/stats.htm

Charrin S, Alcover A. (2006). Role of ERM (ezrin-radixin-moesin) proteins in T lymphocyte polarization, immune synapse formation and in T cell receptor-mediated signaling. Front Biosci. 11:1987-97.

Chaudhry A, Das SR, Hussain A, Mayor S, George A, Bal V, Jameel S, Rath S. (2005). The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. J Immunol. 175(7):4566-74.

Chen JC, Krucinski J, Miercke LJ, Finer-Moore JS, Tang AH, Leavitt AD, Stroud RM. (2000). Crystal structure of the HIV-1 integrase catalytic core and C-terminal domains: a model for viral DNA binding. Proc Natl Acad Sci U S A. 97(15):8233-8.

Chen N, McCarthy C, Drakesmith H, Li D, Cerundolo V, McMichael AJ, Screaton GR, Xu XN. (2005). HIV-1 down-regulates the expression of CD1d via Nef. Eur J Immunol. 36(2):278-286

Chen YL, Trono D, Camaur D. (1998). The proteolytic cleavage of human immunodeficiency virus type 1 Nef does not correlate with its ability to stimulate virion infectivity. J Virol. 72(4):3178-84.

Choi YK, Fallert BA, Murphey-Corb MA, Reinhart TA. (2003). Simian immunodeficiency virus dramatically alters expression of homeostatic chemokines and dendritic cell markers during infection in vivo. Blood. 101(5):1684-91.

Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, Guatelli JC. (1994). Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. J Virol. 68(5):2906-14.

Coffin JM, Hughes SM, Varmus HE. Retroviruses. Plainview (NY): Cold Spring Harbor Laboratory Press; c1997.

Cohen EA[1], Dehni G, Sodroski JG, Haseltine WA. (1990). Human immunodeficiency virus vpr product is a virion-associated regulatory protein. J Virol. 64(6):3097-9.

Cohen EA[2], Terwilliger EF, Jalinoos Y, Proulx J, Sodroski JG, Haseltine WA. (1990). Identification of HIV-1 vpr product and function. J Acquir Immune Defic Syndr. 3:11-8.

Conticello SG, Harris RS, Neuberger MS. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Curr Biol. 13(22):2009-13.

Corless IB, Kirksey KM, Kemppainen J, Nicholas PK, McGibbon C, Davis SM, Dolan S. (2005). Lipodystrophy-associated symptoms and medication adherence in HIV/AIDS. AIDS Patient Care STDS. 19(9):577-86.

Crowe WE, Altamirano AA, Russell JM. (1997). Human cytomegalovirus infection enhances osmotic stimulation of Na+/H+ exchange in human fibroblasts. Am J Physiol. 273(5 Pt 1):C1739-48.

Cujec TP, Okamoto H, Fujinaga K, Meyer J, Chamberlin H, Morgan DO, Peterlin BM. (1997). The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. Genes Dev. 11(20):2645-57.

Cullen BR. (1990). The HIV-1 Tat protein: an RNA sequence-specific processivity factor? Cell. 63(4):655-7.

D'Alfonso S, Richiardi PM. (1994). A polymorphic variation in a putative regulation box of the TNFA promoter region. Immunogenetics. 39(2):150-4.

Daniel R, Kao G, Taganov K, Greger JG, Favorova O, Merkel G, Yen TJ, Katz RA, Skalka AM. (2003). Evidence that the retroviral DNA integration process triggers an ATR-dependent DNA damage response. Proc Natl Acad Sci U S A. 100(8):4778-83.

Dannull J, Surovoy A, Jung G, Moelling K. (1994). Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. EMBO J. 13(7):1525-33.

Datta SR, Brunet A, Greenberg ME. (1999). Cellular survival: a play in three Akts. Genes Dev. 13(22):2905-27.

Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science. 270(5238):988-91.

Decrion AZ, Dichamp I, Varin A, Herbein G. (2005). HIV and inflammation. Curr HIV Res. 3(3):243-59.

Dedera D, Hu W, Vander Heyden N, Ratner L. (1989). Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. J Virol. 63(7):3205-8.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. (1996). Identification of a major co-receptor for primary isolates of HIV-1. Nature. 381(6584):661-6.

Deniaud A, Brenner C, Kroemer G. (2004). Mitochondrial membrane permeabilization by HIV-1 Vpr. Mitochondrion. 4(2-3):223-33.

Depienne C, Roques P, Creminon C, Fritsch L, Casseron R, Dormont D, Dargemont C, Benichou S. (2000). Cellular distribution and karyophilic properties of matrix, integrase, and Vpr proteins from the human and simian immunodeficiency viruses. Exp Cell Res. 260(2):387-95.

Depuydt B, van Loo G, Vandenabeele P, Declercq W. (2005). Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors. J Cell Sci. 118(Pt 3):497-504.

Di Marzio P, Choe S, Ebright M, Knoblauch R, Landau NR. (1995). Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. J Virol. 69(12):7909-16.

Dittmar KD, Banach M, Galigniana MD, Pratt WB. (1998). The role of DnaJ-like proteins in glucocorticoid receptor.hsp90 heterocomplex assembly by the reconstituted hsp90.p60.hsp70 foldosome complex. J Biol Chem. 273(13):7358-66.

Endrich MM, Gehrig P, Gehring H. (1999). Maturation-induced conformational changes of HIV-1 capsid protein and identification of two high affinity sites for cyclophilins in the C-terminal domain. J Biol Chem. 274(9):5326-32.

Elwell LP, Ferone R, Freeman GA, Fyfe JA, Hill JA, Ray PH, Richards CA, Singer SC, Knick VB, Rideout JL, et al. (1987). Antibacterial activity and mechanism of action of 3'-azido-3'-deoxythymidine (BW A509U). Antimicrob Agents Chemother. 31(2):274-80.

Facanha AL, dos Reis MC, Montero-Lomeli M. (2000). Structural study of the porcine Na+/H+ exchanger NHE1 gene and its 5'-flanking region. Mol Cell Biochem. 210(1-2):91-9.

Fang JY, Lu YY. (2002). Effects of histone acetylation and DNA methylation on p21(WAF1) regulation. World J Gastroenterol. 8(3):400-5.

Farnet CM, Haseltine WA. (1991). Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J Virol. 65(4):1910-5.

Faust EA, Triller H. (2002). Stimulation of human flap endonuclease 1 by human immunodeficiency virus type 1 integrase: possible role for flap endonuclease 1 in 5'-end processing of human immunodeficiency virus type 1 integration intermediates. J Biomed Sci. 9(3):273-87.

Felzien LK, Woffendin C, Hottiger MO, Subbramanian RA, Cohen EA, Nabel GJ. (1998). HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 co-activator. Proc Natl Acad Sci U S A. 95(9):5281-6.

Feng S, Holland EC. (1988). HIV-1 tat trans-activation requires the loop sequence within tar. Nature. 334(6178):165-7.

Ferri KF, Jacotot E, Leduc P, Geuskens M, Ingber DE, Kroemer G. (2000). Apoptosis of syncytia induced by the HIV-1-envelope glycoprotein complex: influence of cell shape and size. Exp Cell Res. 261(1):119-26.

Ferri KF, Jacotot E, Blanco J, Este JA, Zamzami N, Susin SA, Xie Z, Brothers G, Reed JC, Penninger JM, Kroemer G. (2000). Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex: role of mitochondria and caspases. J Exp Med. 192(8):1081-92.

Finkel TH, Tudor-Williams G, Banda NK, Cotton MF, Curiel T, Monks C, Baba TW, Ruprecht RM, Kupfer A. (1995). Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat Med. 1(2):129-34.

Fliegel L. (2005). The Na+/H+ exchanger isoform 1. Int J Biochem Cell Biol. 37(1):33-7.

Flori L, Delahaye NF, Iraqi FA, Hernandez-Valladares M, Fumoux F, Rihet P. (2005). TNF as a malaria candidate gene: polymorphism-screening and family-based association analysis of mild malaria attack and parasitemia in Burkina Faso. Genes Immun. 6(6):472-80.

Fritz CC, Green MR. (1996). HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Curr Biol. 6(7):848-54.

Fujita K, Omura S, Silver J. (1997). Rapid degradation of CD4 in cells expressing human immunodeficiency virus type 1 Env and Vpu is blocked by proteasome inhibitors. J Gen Virol. 78 (Pt 3):619-25.

Fukumori T, Akari H, Iida S, Hata S, Kagawa S, Aida Y, Koyama AH, Adachi A. (1998). The HIV-1 Vpr displays strong anti-apoptotic activity. FEBS Lett. 432(1-2):17-20.

Gallo RC. (2002). HISTORICAL ESSAY: The Early Years of HIV/AIDS. Science. 298: 1728-1730.

Ganesh L, Burstein E, Guha-Niyogi A, Louder MK, Mascola JR, Klomp LW, Wijmenga C, Duckett CS, Nabel GJ. (2003). The gene product Murr1 restricts HIV-1 replication in resting CD4+ lymphocytes. Nature. 426(6968):853-7.

Garcia, J. V., and A. D. Miller. (1991). Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 350:508-11.

Geffin R, Wolf D, Muller R, Hill MD, Stellwag E, Freitag M, Sass G, Scott GB, Baur AS. (2000). Functional and structural defects in HIV type 1 nef genes derived from pediatric long-term survivors. AIDS Res Hum Retroviruses. 16(17):1855-68.

Geiss GK, Bumgarner RE, An MC, Agy MB, van 't Wout AB, Hammersmark E, Carter VS, Upchurch D, Mullins JI, Katze MG. (2000). Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays. Virology. 2000. 266(1):8-16.

Goff SP. (1992) Genetics of retroviral integration. Annu. Rev. Genet. 26:527–544.

Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, Hahn BH, Emerman M. R. (1998). HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat Med. 4(1):65-71.

Goh WC, Manel N, Emerman M. (2004). The human immunodeficiency virus Vpr protein binds Cdc25C: implications for G2 arrest. Virology. 318(1):337-49.

Gottlinger HG, Sodroski JG, Haseltine WA. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 86(15):5781-5.

Gragerov A, Kino T, Ilyina-Gragerova G, Chrousos GP, Pavlakis GN. (1998). HHR23A, the human homologue of the yeast repair protein RAD23, interacts specifically with Vpr protein and prevents cell cycle arrest but not the transcriptional effects of Vpr. Virology. 245(2):323-30.

Granelli-Piperno A, Delgado E, Finkel V, Paxton W, Steinman RM. (1998). Immature dendritic cells selectively replicate macrophagetropic (M-tropic) human immunodeficiency virus type 1, while mature cells efficiently transmit both M- and T-tropic virus to T cells. J Virol. 72(4):2733-7.

Guatelli JC. (1997). The positive influence of Nef on viral infectivity. Res Virol. 148(1):34-7.

Ha HC, Juluri K, Zhou Y, Leung S, Hermankova M, Snyder SH. (2001). Poly(ADP-ribose) polymerase-1 is required for efficient HIV-1 integration. Proc Natl Acad Sci U S A. 98(6):3364-8.

Haines DS, Strauss KI, Gillespie DH. (1991). Cellular response to double-stranded RNA. J Cell Biochem. 46(1):9-20.

Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk HD, Garten W. (1992). Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature. 360(6402):358-61.

Hammes SR, Dixon EP, Malim MH, Cullen BR, Greene WC. (1989). Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc Natl Acad Sci U S A. 86(23):9549-53.

Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. (1998). Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell. 95(2):163-75.

Hattori N, Michaels F, Fargnoli K, Marcon L, Gallo RC, Franchini G. (1990). The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. Proc Natl Acad Sci U S A. 87(20):8080-4.

Hauber J, Cullen BR. (1988). Mutational analysis of the trans-activation-responsive region of the human immunodeficiency virus type I long terminal repeat. J Virol. 62(3):673-9.

Haugaard SB, Andersen O, Pedersen SB, Dela F, Fenger M, Richelsen B, Madsbad S, Iversen J. (2006). Tumor necrosis factor alpha is associated with insulin-mediated suppression of free fatty acids and net lipid oxidation in HIV-infected patients with lipodystrophy. Metabolism. 55(2):175-182.

Haughey NJ, Mattson MP. (2002). Calcium dysregulation and neuronal apoptosis by the HIV-1 proteins Tat and gp120. J Acquir Immune Defic Syndr. Suppl 2:S55-61.

He J, Choe S, Walker R, Di Marzio P, Morgan DO, Landau NR. (1995). Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol. 69(11):6705-11.

Health Protection Agency (HPA). HIV and other Sexually Transmitted Infections in the United Kingdom in 2003 Annual Report, November 2004. www.hpa.org.uk/infections/topics_az/hiv_and_sti/publications/annual2004/annual2004.htm

Heckler, M. M. Secretary, U.S. Department of Health and Human services, Washington DC, Statement regarding AIDS, April 23, 1984

Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, Gendelman HE, Ratner L, Stevenson M, Emerman M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc Natl Acad Sci U S A. 91(15):7311-5.

Henderson, L. E., R. C. Sowder, T. D. Copeland, S. Oroszlan, and R. E. Benveniste. (1990). Gag precursors of HIV and SIV are cleaved into six proteins found in the mature virions. J. Med. Primatol. 19:411-419

Herbein G, Van Lint C, Lovett JL, Verdin E. (1998). Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J Virol. 72(1):660-70.

Holm GH, Gabuzda D. (2005). Distinct mechanisms of CD4+ and CD8+ T-cell activation and bystander apoptosis induced by human immunodeficiency virus type 1 virions. J Virol. 79(10):6299-311.

Horvat RT, Wood C. (1989). HIV promoter activity in primary antigen-specific human T lymphocytes. J Immunol. 143(8):2745-51.

Hrecka K, Swigut T, Schindler M, Kirchhoff F, Skowronski J. (2005). Nef proteins from diverse groups of primate lentiviruses downmodulate CXCR4 to inhibit migration to the chemokine stromal derived factor 1. J Virol. 79(16):10650-9.

Hu J, Li B, Kihara D. (2005). Limitations and potentials of current motif discovery algorithms. Nucleic Acids Res. 33(15):4899-913.

Huang Y, Erdmann N, Zhao J, Zheng J. (2005). The signaling and apoptotic effects of TNF-related apoptosis-inducing ligand in HIV-1 associated dementia. Neurotox Res. 8(1-2):135-48.

Isel C, Karn J. (1999). Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation. J Mol Biol. 290(5):929-41.

Jacotot E, Ravagnan L, Loeffler M, Ferri KF, Vieira HL, Zamzami N, Costantini P, Druillennec S, Hoebeke J, Briand JP, Irinopoulou T, Daugas E, Susin SA, Cointe D, Xie ZH, Reed JC, Roques BP, Kroemer G. (2000). The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J Exp Med. 191(1):33-46.

Jacotot E, Ferri KF, El Hamel C, Brenner C, Druillennec S, Hoebeke J, Rustin P, Metivier D, Lenoir C, Geuskens M, Vieira HL, Loeffler M, Belzacq AS, Briand JP, Zamzami N, Edelman L, Xie ZH, Reed JC, Roques BP, Kroemer G. (2001). Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2. J Exp Med. 2001 Feb 19;193(4):509-19.

Janket ML, Manickam P, Majumder B, Thotala D, Wagner M, Schafer EA, Collman RG, Srinivasan A, Ayyavoo V. (2004). Differential regulation of host cellular genes by HIV-1 viral protein R (Vpr): cDNA microarray analysis using isogenic virus. Biochem Biophys Res Commun. 314(4):1126-32.

Janknecht R. (2002). The versatile functions of the transcriptional coactivators p300 and CBP and their roles in disease. Histol Histopathol. 17(2):657-68.

Jenkins Y, McEntee M, Weis K, Greene WC. (1998). Characterization of HIV-1 vpr nuclear import: analysis of signals and pathways. J Cell Biol. 1998 Nov 16;143(4):875-85.

Jian H, Zhao LJ. (2003). Pro-apoptotic activity of HIV-1 auxiliary regulatory protein Vpr is subtype-dependent and potently enhanced by nonconservative changes of the leucine residue at position 64. J Biol Chem. 278(45):44326-30.

Joseph AM, Kumar M, Mitra D. (2005). Nef: "necessary and enforcing factor" in HIV infection. Curr HIV Res. 3(1):87-94.

Jowett JB, Planelles V, Poon B, Shah NP, Chen ML, Chen IS. (1995). The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J Virol. 69(10):6304-13.

Kahler AK, Persson AS, Sanchez F, Kallstrom H, Apt AS, Schurr E, Lavebratt C. (2005). A new coding mutation in the Tnf-alpha leader sequence in tuberculosis-sensitive I/St mice causes higher secretion levels of soluble TNF-alpha. Genes Immun. 6(7):620-7.

Kaiser Family Foundation. Fact Sheet: U.S. Federal Funding for HIV/AIDS: The FY 2006 Budget Request, February 2005.

Kalkhoven E. (2004). CBP and p300: HATs for different occasions. Biochem Pharmacol. 2004 68(6):1145-55.

Karczewski MK, Strebel K. (1996). Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. J Virol. 70(1):494-507.

Kato H, Sumimoto H, Pognonec P, Chen CH, Rosen CA, Roeder RG. (1992). HIV-1 Tat acts as a processivity factor in vitro in conjunction with cellular elongation factors. Genes Dev. 6(4):655-66.

Khamsri B, Murao F, Yoshida A, Sakurai A, Uchiyama T, Shirai H, Matsuo Y, Fujita M, Adachi A. (2006). Comparative study on the structure and cytopathogenic activity of HIV Vpr/Vpx proteins. Microbes Infect. 8(1):10-5.

Kim S, Ikeuchi K, Byrn R, Groopman J, Baltimore D. (1989). Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 86(23):9544-8.

Kim YK, Bourgeois CF, Isel C, Churcher MJ, Karn J. (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. Mol Cell Biol. 22(13):4622-37.

Kino T, Gragerov A, Kopp JB, Stauber RH, Pavlakis GN, Chrousos GP. (1999). The HIV-1 virion-associated protein vpr is a coactivator of the human glucocorticoid receptor. J Exp Med. 189(1):51-62.

Kino T, Gragerov A, Slobodskaya O, Tsopanomichalou M, Chrousos GP, Pavlakis GN. (2002). Human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators. J Virol. 76(19):9724-34.

Kino T, Tsukamoto M, Chrousos G. (2002). Transcription factor TFIIH components enhance the GR coactivator activity but not the cell cycle-arresting activity of the human immunodeficiency virus type-1 protein Vpr. Biochem Biophys Res Commun. 298(1):17-23.

Kino T, De Martino MU, Charmandari E, Mirani M, Chrousos GP. (2003). Tissue glucocorticoid resistance/hypersensitivity syndromes. J Steroid Biochem Mol Biol. 85(2-5):457-67.

Kino T, Chrousos GP. (2004). Human immunodeficiency virus type-1 accessory protein Vpr: a causative agent of the AIDS-related insulin resistance/lipodystrophy syndrome? Ann N Y Acad Sci. 1024:153-67.

Kino T, Gragerov A, Valentin A, Tsopanomihalou M, Ilyina-Gragerova G, Erwin-Cohen R, Chrousos GP, Pavlakis GN. (2005). Vpr protein of human immunodeficiency virus type 1 binds to 14-3-3 proteins and facilitates complex formation with Cdc25C: implications for cell cycle arrest. J Virol. 79(5):2780-7.

Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein. (1990). The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. J Virol 64:621-9.

Knight JC, Udalova I, Hill AV, Greenwood BM, Peshu N, Marsh K, Kwiatkowski D. (1999). A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet. 22(2):145-50.

Kocsis J, Prohaszka Z, Biro A, Fust G, Banhegyi D. (2003). Elevated levels of antibodies against 70 kDa heat shock proteins in the sera of patients with HIV infection.. J Med Virol. 71(4):480-2.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science. 256: 1783-1790.

Kolesnitchenko V, Wahl LM, Tian H, Sunila I, Tani Y, Hartmann DP, Cossman J, Raffeld M, Orenstein J, Samelson LE, Cohen DI. (1995). Human immunodeficiency virus 1 envelope-initiated G2-phase programmed cell death. Proc Natl Acad Sci U S A. 92(25):11889-93.

Kolluri SK, Bruey-Sedano N, Cao X, Lin B, Lin F, Han YH, Dawson MI, Zhang XK. (2003). Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. Mol Cell Biol. 23(23):8651-67.

Kondo E, Mammano F, Cohen EA, Gottlinger HG. (1995). The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J Virol. 69(5):2759-64.

Kornbluth RS, Rhodes J, Richman DD. (1993). Recovery of infectious HIV following the passage of apoptotic cellular debris through phagocytic macrophages. Chest. 103(2 Suppl):102S-103S.

Lama J, Mangasarian A, Trono D. (1999). Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr Biol. 9(12):622-31.

Lasky LA, Nakamura G, Smith DH, Fennie C, Shimasaki C, Patzer E, Berman P, Gregory T, Capon DJ. (1987). Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell. 50(6):975-85.

Laurent-Crawford AG, Krust B, Riviere Y, Desgranges C, Muller S, Kieny MP, Dauguet C, Hovanessian AG. (1993). Membrane expression of HIV envelope glycoproteins triggers apoptosis in CD4 cells. AIDS Res Hum Retroviruses. 9(8):761-73.

Leo C, Chen JD. (2000). The SRC family of nuclear receptor coactivators. Gene. 245(1):1-11.

Le Rouzic E, Mousnier A, Rustum C, Stutz F, Hallberg E, Dargemont C, Benichou S. (2002). Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. J Biol Chem. 277(47):45091-8.

Levesque K, Zhao YS, Cohen EA. (2003). Vpu exerts a positive effect on HIV-1 infectivity by down-modulating CD4 receptor molecules at the surface of HIV-1-producing cells. J Biol Chem. 278(30):28346-53.

Levy DN, Fernandes LS, Williams WV, Weiner DB. (1993). Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell. 72(4):541-50.

Levy DN, Refaeli Y, MacGregor RR, Weiner DB. (1994). Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 91(23):10873-7.

Li Y, Sarkar FH. (2002). Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett. 186(2):157-64.

Lin YL, Mettling C, Portales P, Reynes J, Clot J, Corbeau P. (2002). Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection. Proc Natl Acad Sci U S A. 26;99(24):15590-5.

Liu H, Wu X, Newman M, Shaw GM, Hahn BH, Kappes JC. (1995). The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. J Virol. 69(12):7630-8.

Lossos IS, Czerwinski DK, Wechser MA, Levy R. (2003). Optimization of quantitative real-time RT-PCR parameters for the study of lymphoid malignancies. Leukemia. 17(4):789-95.

Lu YY, Koga Y, Tanaka K, Sasaki M, Kimura G, Nomoto K. (1994). Apoptosis induced in CD4+ cells expressing gp160 of human immunodeficiency virus type 1. J Virol. 1994 68(1):390-9.

Macreadie IG, Castelli LA, Hewish DR, Kirkpatrick A, Ward AC, Azad AA. (1995). A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects. Proc Natl Acad Sci U S A. 92(7):2770-4.

Macreadie IG, Arunagiri CK, Hewish DR, White JF, Azad AA. (1996). Extracellular addition of a domain of HIV-1 Vpr containing the amino acid sequence motif H(S/F)RIG causes cell membrane permeabilization and death. Mol Microbiol. 19(6):1185-92.

Madani, N., and D. Kabat. (1998). An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. J Virol 72:10251-5.

Mahalingam S, Ayyavoo V, Patel M, Kieber-Emmons T, Weiner DB. (1997). Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. J Virol. 71(9):6339-47.

Mahalingam S, MacDonald B, Ugen KE, Ayyavoo V, Agadjanyan MG, Williams WV, Weiner DB. (1997). In vitro and in vivo tumor growth suppression by HIV-1 Vpr. DNA Cell Biol. 16(2):137-43.

Maher B, Alfirevic A, Vilar FJ, Wilkins EG, Park BK, Pirmohamed M. (2002). TNF-alpha promoter region gene polymorphisms in HIV-positive patients with lipodystrophy. AIDS. 16(15):2013-8.

Majumder B, Janket ML, Schafer EA, Schaubert K, Huang XL, Kan-Mitchell J, Rinaldo CR Jr, Ayyavoo V. (2005). Human immunodeficiency virus type 1 Vpr impairs dendritic cell maturation and T-cell activation: implications for viral immune escape. J Virol. 2005 79(13):7990-8003.

Mandell CP, Reyes RA, Cho K, Sawai ET, Fang AL, Schmidt KA, Luciw PA. (1999). SIV/HIV Nef recombinant virus (SHIVnef) produces simian AIDS in rhesus macaques. Virology. 265(2):235-51.

Mangelsdorf DJ, Evans RM. (1995). The RXR heterodimers and orphan receptors. Cell. 83(6):841-50.

Maragos WF, Tillman P, Jones M, Bruce-Keller AJ, Roth S, Bell JE, Nath A. (2003). Neuronal injury in hippocampus with human immunodeficiency virus transactivating protein, Tat. Neuroscience. 117(1):43-53.

Mark-Danieli M, Laham N, Kenan-Eichler M, Castiel A, Melamed D, Landau M, Bouvier NM, Evans MJ, Bacharach E. (2005). Single point mutations in the zinc finger motifs of the human immunodeficiency virus type 1 nucleocapsid alter RNA binding specificities of the gag protein and enhance packaging and infectivity. J Virol. 79(12):7756-67.

Marsh JW. (1999). The numerous effector functions of Nef. Arch Biochem Biophys. 365:192-8.

Masuda M, Nagai Y, Oshima N, Tanaka K, Murakami H, Igarashi H, Okayama H. (2000). Genetic studies with the fission yeast Schizosaccharomyces pombe suggest involvement of wee1, ppa2, and rad24 in induction of cell cycle arrest by human immunodeficiency virus type 1 Vpr. J Virol. 74(6):2636-46.

Mathe G. (1999). Why have ten or so nontoxic, retrovirus integrase inhibitors not been made available for AIDS treatment? A ten-year experience must liberate them. Biomed Pharmacother. 53(10):484-6.

Mazumder A, Engelman A, Craigie R, Fesen M, Pommier Y. (1994). Intermolecular disintegration and intramolecular strand transfer activities of wild-type and mutant HIV-1 integrase. Nucleic Acids Res. 22(6):1037-43.

Mehle A, Strack B, Ancuta P, Zhang C, McPike M, Gabuzda D. (2004). Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. J Biol Chem. 279(9):7792-8.

Miles MC, Janket ML, Wheeler ED, Chattopadhyay A, Majumder B, Dericco J, Schafer EA, Ayyavoo V. (2005). Molecular and functional characterization of a novel splice variant of ANKHD1 that lacks the KH domain and its role in cell survival and apoptosis. FEBS J. 272(16):4091-102.

Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB. (1994). The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J Exp Med. 179(1):101-13.

Miller MD, Warmerdam MT, Page KA, Feinberg MB, Greene WC. (1995). Expression of the human immunodeficiency virus type 1 (HIV-1) nef gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. J Virol. 69(1):579-84.

Miller MD, Farnet CM, Bushman FD. (1997). Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J Virol. 71(7):5382-90.

Miller MD, Warmerdam MT, Ferrell SS, Benitez R, Greene WC. (1997). Intravirion generation of the C-terminal core domain of HIV-1 Nef by the HIV-1 protease is insufficient to enhance viral infectivity. Virology. 234(2):215-25.

Mirani M, Elenkov I, Volpi S, Hiroi N, Chrousos GP, Kino T. (2002). HIV-1 protein Vpr suppresses IL-12 production from human monocytes by enhancing glucocorticoid action: potential implications of Vpr coactivator activity for the innate and cellular immunity deficits observed in HIV-1 infection. J Immunol. 169(11):6361-8.

Morellet N, Bouaziz S, Petitjean P, Roques BP. (2003). NMR structure of the HIV-1 regulatory protein VPR. J Mol Biol. 327(1):215-27.

Mulder LC, Chakrabarti LA, Muesing MA. (2002). Interaction of HIV-1 integrase with DNA repair protein hRad18. J Biol Chem. 277(30):27489-93.

Muthumani K, Hwang DS, Desai BM, Zhang D, Dayes N, Green DR, Weiner DB. (2002). HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. J Biol Chem. 277(40):37820-31.

Muthumani K, Choo AY, Zong WX, Madesh M, Hwang DS, Premkumar A, Thieu KP, Emmanuel J, Kumar S, Thompson CB, Weiner DB. (2006). The HIV-1 Vpr and

glucocorticoid receptor complex is a gain-of-function interaction that prevents the nuclear localization of PARP-1. Nat Cell Biol. 8(2):170-9.

Muto S, Ebata S, Okada K, Saito T, Asano Y. (2000). Glucocorticoid modulates Na+/H+ exchange activity in vascular smooth muscle cells by nongenomic and genomic mechanisms. Kidney Int. 57(6):2319-33.

Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D. (1986). Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med. 163(6):1363-75.

Niederman TM, Thielan BJ, Ratner L. (1989). Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci U S A. 86(4):1128-32.

Nishizawa M, Kamata M, Mojin T, Nakai Y, Aida Y. (2000). Induction of apoptosis by the Vpr protein of human immunodeficiency virus type 1 occurs independently of G(2) arrest of the cell cycle. Virology. 276(1):16-26.

Okada H, Takei R, Tashiro M. (1997). Nef protein of HIV-1 induces apoptotic cytolysis of murine lymphoid cells independently of CD95 (Fas) and its suppression by serine/threonine protein kinase inhibitors. FEBS Lett. 417(1):61-4.

Pandori MW, Fitch NJ, Craig HM, Richman DD, Spina CA, Guatelli JC. (1996). Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J Virol. 70(7):4283-90.

Pang S, Kang MK, Kung S, Yu D, Lee A, Poon B, Chen IS, Lindemann B, Park NH. (2001). Anticancer effect of a lentiviral vector capable of expressing HIV-1 Vpr. Clin Cancer Res. 7(11):3567-73.

Paul M, Jabbar MA. (1997). Phosphorylation of both phosphoacceptor sites in the HIV-1 Vpu cytoplasmic domain is essential for Vpu-mediated ER degradation of CD4. Virology. 232(1):207-16.

Paxton W, Connor RI, Landau NR. (1993). Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. J Virol. 67(12):7229-37.

Pedersen AG, Baldi P, Chauvin Y, Brunak S. (1999). The biology of eukaryotic promoter prediction--a review. Comput Chem. 1999 Jun 15;23(3-4):191-207.

Peterlin BM, Trono D. (2003). Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol. 3(2):97-107.

Pettit SC, Everitt LE, Choudhury S, Dunn BM, Kaplan AH. (2004). Initial cleavage of the human immunodeficiency virus type 1 GagPol precursor by its activated protease occurs by an intramolecular mechanism. J Virol. 78(16):8477-85.

Pettit SC, Clemente JC, Jeung JA, Dunn BM, Kaplan AH. (2005). Ordered processing of the human immunodeficiency virus type 1 GagPol precursor is influenced by the context of the embedded viral protease. J Virol. 79(16):10601-7.

Philippon V, Matsuda Z, Essex M. (1999). Transactivation is a conserved function among primate lentivirus Vpr proteins but is not shared by Vpx. J Hum Virol. 2(3):167-74.

Piguet V, Schwartz O, Le Gall S, Trono D. (1999). The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol Rev. 168:51-63.

Piguet V, Wan L, Borel C, Mangasarian A, Demaurex N, Thomas G, Trono D. (2000). HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. Nat Cell Biol. 2(3):163-7.

Planelles V, Jowett JB, Li QX, Xie Y, Hahn B, Chen IS. (1996). Vpr-induced cell cycle arrest is conserved among primate lentiviruses. J Virol. 70(4):2516-24.

Pober JS. (1987). Effects of tumour necrosis factor and related cytokines on vascular endothelial cells. Ciba Found Symp. 131:170-84.

Pomerantz RJ. (2004). Effects of HIV-1 Vpr on neuroinvasion and neuropathogenesis. DNA Cell Biol. 23(4):227-38.

Poon B, Chen IS. (2003). Human immunodeficiency virus type 1 (HIV-1) Vpr enhances expression from unintegrated HIV-1 DNA. J Virol. 77(7):3962-72.

Popovic M, Sarngadharan MG, Read E, Gallo RC. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science. 224(4648):497-500.

Popov S, Rexach M, Ratner L, Blobel G, Bukrinsky M. (1998). Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. J Biol Chem. 273(21):13347-52.

Pu H, Tian J, Flora G, Lee YW, Nath A, Hennig B, Toborek M. (2003). HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain. Mol Cell Neurosci. 24(1):224-37.

Pudney J, Song MJ. (1994). Electron microscopic analysis of HIV-host cell interactions. Tissue Cell. 26(4):539-50.

Putney LK, Barber DL. (2003). Na-H exchange-dependent increase in intracellular pH times G2/M entry and transition. J Biol Chem. 278(45):44645-9.

Putney LK, Denker SP, Barber DL. (2002). The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions. Annu Rev Pharmacol Toxicol. 42:527-52.

Putney LK, Barber DL. (2003). Na-H exchange-dependent increase in intracellular pH times G2/M entry and transition. J Biol Chem. 278(45):44645-9.

Qiu P. (2003). Recent advances in computational promoter analysis in understanding the transcriptional regulatory network. Biochem Biophys Res Commun. 2003 Sep 26;309(3):495-501.

Ramanathan MP, Curley E 3rd, Su M, Chambers JA, Weiner DB. (2002). Carboxyl terminus of hVIP/mov34 is critical for HIV-1-Vpr interaction and glucocorticoid-mediated signaling. J Biol Chem. 277(49):47854-60.

Rasola A, Gramaglia D, Boccaccio C, Comoglio PM. (2001). Apoptosis enhancement by the HIV-1 Nef protein. J Immunol. 166(1):81-8.

Re F, Braaten D, Franke EK, Luban J. (1995). Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. J Virol. 69(11):6859-64.

Refaeli Y, Levy DN, Weiner DB. (1995). The glucocorticoid receptor type II complex is a target of the HIV-1 vpr gene product. Proc Natl Acad Sci U S A. 92(8):3621-5.

Rich IN, Worthington-White D, Garden OA, Musk P. (2000). Apoptosis of leukemic cells accompanies reduction in intracellular pH after targeted inhibition of the Na(+)/H(+) exchanger. 95(4):1427-34.

Rogel ME, Wu LI, Emerman M. (1995). The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. J Virol. 1995 Feb;69(2):882-8.

Rossio JL, Esser MT, Suryanarayana K, Schneider DK, Bess JW Jr, Vasquez GM, Wiltrout TA, Chertova E, Grimes MK, Sattentau Q, Arthur LO, Henderson LE, Lifson JD. (1998). Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins. J Virol. 72(10):7992-8001.

Roumier T, Vieira HL, Castedo M, Ferri KF, Boya P, Andreau K, Druillennec S, Joza N, Penninger JM, Roques B, Kroemer G. (2002). The C-terminal moiety of HIV-1 Vpr induces cell death via a caspase-independent mitochondrial pathway. Cell Death Differ. 2002. 9(11):1212-9.

Roux P, Alfieri C, Hrimech M, Cohen EA, Tanner JE. (2000). Activation of transcription factors NF-kappaB and NF-IL-6 by human immunodeficiency virus type 1 protein R (Vpr) induces interleukin-8 expression. J Virol. 74(10):4658-65.

Rusert P, Fischer M, Joos B, Leemann C, Kuster H, Flepp M, Bonhoeffer S, Gunthard HF, Trkola A. (2004). Quantification of infectious HIV-1 plasma viral load using a boosted in vitro infection protocol. Virology. 326(1):113-29.

Safrin S, Grunfeld C. (1999). Fat distribution and metabolic changes in patients with HIV infection. AIDS. 13(18):2493-505.

Sallakci N, Akcurin G, Koksoy S, Kardelen F, Uguz A, Coskun M, Ertug H, Yegin O. (2005). TNF-alpha G-308A polymorphism is associated with rheumatic fever and correlates with increased TNF-alpha production. J Autoimmun. 25(2):150-4.

Sawaya BE, Khalili K, Rappaport J, Serio D, Chen W, Srinivasan A, Amini S. (1999). Suppression of HIV-1 transcription and replication by a Vpr mutant. Gene Ther. 6(5):947-50.

Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med. 3(11):1259-65.

Schaefer TM, Bell I, Pfeifer ME, Ghosh M, Trible RP, Fuller CL, Ashman C, Reinhart TA. (2002). The conserved process of TCR/CD3 complex down-modulation by SIV Nef is mediated by the central core, not endocytic motifs. Virology. 302(1):106-22.

Schatz O, Mous J, Le Grice SF. (1990). HIV-1 RT-associated ribonuclease H displays both endonuclease and 3'----5' exonuclease activity. EMBO J. 9(4):1171-6.

Schindler M, Wurfl S, Benaroch P, Greenough TC, Daniels R, Easterbrook P, Brenner M, Munch J, Kirchhoff F. (2003). Down-modulation of mature major histocompatibility complex class II and up-regulation of invariant chain cell surface expression are well-conserved functions of human and simian immunodeficiency virus nef alleles. J Virol. 77(19):10548-56.

Schneider D, Gerhardt E, Bock J, Muller MM, Wolburg H, Lang F, Schulz JB. (2004). Intracellular acidification by inhibition of the Na+/H+-exchanger leads to caspase-independent death of cerebellar granule neurons resembling paraptosis. Cell Death Differ. 11(7):760-70.

Schuler W, Wecker K, de Rocquigny H, Baudat Y, Sire J, Roques BP. (1999). NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: molecular insights into its biological functions. J Mol Biol. 285(5):2105-17.

Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J. M. Heard. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med 2:338-42.

Sheehy AM, Gaddis NC, Choi JD, Malim MH. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature. 418(6898):646-50.

Sheehy AM, Gaddis NC, Malim MH. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nat Med. 9(11):1404-7.

Sherman MP, de Noronha CM, Pearce D, Greene WC. (2000). Human immunodeficiency virus type 1 Vpr contains two leucine-rich helices that mediate glucocorticoid receptor coactivation independently of its effects on G(2) cell cycle arrest. J Virol. 74(17):8159-65.

Sherman MP, de Noronha CM, Heusch MI, Greene S, Greene WC. (2001). Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr. J Virol. 75(3):1522-32.

Sherman MP, de Noronha CM, Eckstein LA, Hataye J, Mundt P, Williams SA, Neidleman JA, Goldsmith MA, Greene WC. (2003). Nuclear export of Vpr is required for efficient replication of human immunodeficiency virus type 1 in tissue macrophages. J Virol. 77(13):7582-9.

Shi B, Raina J, Lorenzo A, Busciglio J, Gabuzda D. (1998). Neuronal apoptosis induced by HIV-1 Tat protein and TNF-alpha: potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia. J Neurovirol. 1998 Jun;4(3):281-90.

Shostak LD, Ludlow J, Fisk J, Pursell S, Rimel BJ, Nguyen D, Rosenblatt JD, Planelles V. (1999). Roles of p53 and caspases in the induction of cell cycle arrest and apoptosis by HIV-1 vpr. Exp Cell Res. 251(1):156-65.

Simard MC, Chrobak P, Kay DG, Hanna Z, Jothy S, Jolicoeur P. (2002). Expression of simian immunodeficiency virus nef in immune cells of transgenic mice leads to a severe AIDS-like disease. J Virol. 76(8):3981-95.

Simon JH[1], Gaddis NC, Fouchier RA, Malim MH. (1998). Evidence for a newly discovered cellular anti-HIV-1 phenotype. Nat Med. 4(12):1397-400.

Simon JH[2], Miller DL, Fouchier RA, Soares MA, Peden KW, Malim MH. (1998). The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission. EMBO J. 17(5):1259-67.

Smith PD, Meng G, Sellers MT, Rogers TS, Shaw GM. (2000). Biological parameters of HIV-1 infection in primary intestinal lymphocytes and macrophages. J Leukoc Biol. 68(3):360-5.

Sorensen JB, Fernandez-Chacon R, Sudhof TC, Neher E. (2003). Examining synaptotagmin 1 function in dense core vesicle exocytosis under direct control of Ca2+. J Gen Physiol. 122(3):265-76.

Soriano V, de Mendoza C. (2002). Genetic mechanisms of resistance to NRTI and NNRTI. HIV Clin Trials. 3(3):237-48.

Starnes MC, Cheng YC. (1989). Human immunodeficiency virus reverse transcriptase-associated RNase H activity. J Biol Chem. 264(12):7073-7.

Stewart SA, Poon B, Song JY, Chen IS. (2000). Human immunodeficiency virus type 1 vpr induces apoptosis through caspase activation. J Virol. 74(7):3105-11.

Stopak K, de Noronha C, Yonemoto W, Greene WC. (2003). HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol Cell. 12(3):591-601.

Stopak K, Greene WC. (2005). Protecting APOBEC3G: a potential new target for HIV drug discovery. Curr Opin Investig Drugs. 6(2):141-7.

Stove V, Van de Walle I, Naessens E, Coene E, Stove C, Plum J, Verhasselt B. (2005). Human immunodeficiency virus Nef induces rapid internalization of the T-cell coreceptor CD8alphabeta. J Virol. 79(17):11422-33.

Swigut T, Shohdy N, Skowronski J. (2001). Mechanism for down-regulation of CD28 by Nef. EMBO J. 20(7):1593-604.

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

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A., and Arnold, E. (1994) Locations of Anti-AIDS Drug Binding Sites and Resistance Mutations in the Three-dimensional Structure of HIV-1 Reverse Transcriptase: Implications for Mechanisms of Drug Inhibition and Resistance. J. Mol. Biol. 243: 369-387

Thotala D, Schafer EA, Tungaturthi PK, Majumder B, Janket ML, Wagner M, Srinivasan A, Watkins S, Ayyavoo V.(2004). Structure-functional analysis of human immunodeficiency virus type 1 (HIV-1) Vpr: role of leucine residues on Vpr-mediated transactivation and virus replication. Virology. 328(1):89-100.

Tolstrup M, Ostergaard L, Laursen AL, Pedersen SF, Duch M. (2004). HIV/SIV escape from immune surveillance: focus on Nef. Curr HIV Res. 2(2):141-51.

Towers GJ, Hatziioannou T, Cowan S, Goff SP, Luban J, Bieniasz PD. (2003). Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat Med. 9(9):1138-43.

Toy EP, Rodriguez-Rodriguez L, McCance D, Ludlow J, Planelles V. (2000). Induction of cell-cycle arrest in cervical cancer cells by the human immunodeficiency virus type 1 viral protein R. Obstet Gynecol. 95(1):141-6.

Trono D. (1995). HIV accessory proteins: leading roles for the supporting cast. Cell. 82(2):189-92.

Tungaturthi PK, Sawaya BE, Singh SP, Tomkowicz B, Ayyavoo V, Khalili K, Collman RG, Amini S, Srinivasan A. (2003). Role of HIV-1 Vpr in AIDS pathogenesis: relevance and implications of intravirion, intracellular and free Vpr. Biomed Pharmacother. 57(1):20-4.

Ubalee R, Suzuki F, Kikuchi M, Tasanor O, Wattanagoon Y, Ruangweerayut R, Na-Bangchang K, Karbwang J, Kimura A, Itoh K, Kanda T, Hirayama K. (2001). Strong association of a tumor necrosis factor-alpha promoter allele with cerebral malaria in Myanmar. Tissue Antigens. 58(6):407-10.

UNAIDS. (2004) AIDS Epidemic Update , December 2004. www.unaids.org/wad2004/report_pdf.html

Vahey MT, Nau ME, Jagodzinski LL, Yalley-Ogunro J, Taubman M, Michael NL, Lewis MG. (2002). Impact of viral infection on the gene expression profiles of proliferating normal human peripheral blood mononuclear cells infected with HIV type 1 RF. AIDS Res Hum Retroviruses. 18(3):179-92.

Vanitharani R, Mahalingam S, Rafaeli Y, Singh SP, Srinivasan A, Weiner DB, Ayyavoo V. (2001). HIV-1 Vpr transactivates LTR-directed expression through sequences present within -278 to -176 and increases virus replication in vitro. Virology. 289(2):334-42.

van 't Wout AB, Lehrman GK, Mikheeva SA, O'Keeffe GC, Katze MG, Bumgarner RE, Geiss GK, Mullins JI. (2003). Cellular gene expression upon human immunodeficiency virus type 1 infection of CD4(+)-T-cell lines. J Virol. 77(2):1392-402.

Varin A, Decrion AZ, Sabbah E, Quivy V, Sire J, Van Lint C, Roques BP, Aggarwal BB, Herbein G. (2005). Synthetic Vpr protein activates activator protein-1, c-Jun N-terminal kinase, and NF-kappaB and stimulates HIV-1 transcription in promonocytic cells and primary macrophages. J Biol Chem. 280(52):42557-67.

Vieira HL, Haouzi D, El Hamel C, Jacotot E, Belzacq AS, Brenner C, Kroemer G. (2000). Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ. 7(12):1146-54.

Verrier F, Borman AM, Brand D, Girard M. (1999). Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. AIDS Res Hum Retroviruses. 15(8):731-43.

Vivet-Boudou V, Didierjean J, Isel C, Marquet R. (2006). Nucleoside and nucleotide inhibitors of HIV-1 replication. Cell Mol Life Sci. 63(2):163-186.

Viraben R, Aquilina C. (1998). Indinavir-associated lipodystrophy. AIDS. 12(6):F37-9.

von Schwedler U, Kornbluth RS, Trono D. (1994). The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc Natl Acad Sci U S A. 91(15):6992-6.

Wang JY, Ling H, Yang W, Craigie R. (2001). Structure of a two-domain fragment of HIV-1 integrase: implications for domain organization in the intact protein. EMBO J. 20(24):7333-43.

Wang L, Mukherjee S, Jia F, Narayan O, Zhao LJ. (1995). Interaction of virion protein Vpr of human immunodeficiency virus type 1 with cellular transcription factor Sp1 and trans-activation of viral long terminal repeat. J Biol Chem. 270(43):25564-9.

Wang L, Mukherjee S, Narayan O, Zhao LJ. (1996). Characterization of a leucine-zipper-like domain in Vpr protein of human immunodeficiency virus type 1. Gene. 178(1-2):7-13

Warren AJ. (2002). Eukaryotic transcription factors. Curr Opin Struct Biol. 12(1):107-14.

Wecker K, Roques BP. (1999). NMR structure of the (1-51) N-terminal domain of the HIV-1 regulatory protein Vpr. Eur J Biochem. 266(2):359-69.

Welker R, Kottler H, Kalbitzer HR, Krausslich HG. (1996). Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology. 219(1):228-36.

Wiegand HL, Doehle BP, Bogerd HP, Cullen BR. (2004). A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. EMBO J. 23:2451-8.

Wen W, Meinkoth JL, Tsien RY, Taylor SS. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell. 82(3):463-73.

Wieder ED, Hang H, Fox MH. (1993) Measurement of intracellular pH using flow cytometry with carboxy-SNARF-1. Cytometry. 14(8):916-21.

Willetts KE, Rey F, Agostini I, Navarro JM, Baudat Y, Vigne R, Sire J. (1999). DNA repair enzyme uracil DNA glycosylase is specifically incorporated into human immunodeficiency virus type 1 viral particles through a Vpr-independent mechanism. J Virol. 73(2):1682-8.

Willey RL, Maldarelli F, Martin MA, Strebel K. (1992). Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J Virol. 66(12):7193-200.

Williams GT, Lau LF. (1993). Activation of the inducible orphan receptor gene nur77 by serum growth factors: dissociation of immediate-early and delayed-early responses. Mol Cell Biol. 13(10):6124-36.

Withers-Ward ES, Jowett JB, Stewart SA, Xie YM, Garfinkel A, Shibagaki Y, Chow SA, Shah N, Hanaoka F, Sawitz DG, Armstrong RW, Souza LM, Chen IS. (1997). Human immunodeficiency virus type 1 Vpr interacts with HHR23A, a cellular protein implicated in nucleotide excision DNA repair. J Virol. 71(12):9732-42.

Wu KL, Khan S, Lakhe-Reddy S, Jarad G, Mukherjee A, Obejero-Paz CA, Konieczkowski M, Sedor JR, Schelling JR. (2004). The NHE1 Na+/H+ exchanger recruits ezrin/radixin/moesin proteins to regulate Akt-dependent cell survival. J Biol Chem. 279(25):26280-6.

Xiao L, Owen SM, Goldman I, Lal AA, deJong JJ, Goudsmit J, Lal RB. (1998). CCR5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage. Virology. 240(1):83-92.

Xiao W, Oefner PJ. (2001). Denaturing high-performance liquid chromatography: A review. Hum Mutat. 17(6):439-74.

Xu XN, Laffert B, Screaton GR, Kraft M, Wolf D, Kolanus W, Mongkolsapay J, McMichael AJ, Baur AS. (1999). Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J Exp Med. 189(9):1489-96.

Yamashita K, Takahashi A, Kobayashi S, Hirata H, Mesner PW Jr, Kaufmann SH, Yonehara S, Yamamoto K, Uchiyama T, Sasada M. (1999). Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and downregulation of reactive oxygen production. Blood. 93(2):674-85.

Yang Y, Tikhonov I, Ruckwardt TJ, Djavani M, Zapata JC, Pauza CD, Salvato MS. (2003). Monocytes treated with human immunodeficiency virus Tat kill uninfected CD4(+) cells by a tumor necrosis factor-related apoptosis-induced ligand-mediated mechanism. J Virol. 77(12):6700-8.

Yasuda J, Miyao T, Kamata M, Aida Y, Iwakura Y. (2001). T cell apoptosis causes peripheral T cell depletion in mice transgenic for the HIV-1 vpr gene. Virology. 285(2):181-92.

Yedavalli VS, Shih HM, Chiang YP, Lu CY, Chang LY, Chen MY, Chuang CY, Dayton AI, Jeang KT, Huang LM. (2005). Human immunodeficiency virus type 1 Vpr interacts with antiapoptotic mitochondrial protein HAX-1. J Virol. 79(21):13735-46.

Yu X, Yuan X, Matsuda Z, Lee TH, Essex M. (2002). The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J Virol. 66(8):4966-71.

Yuan H, Xie YM, Chen IS. (2003). Depletion of Wee-1 kinase is necessary for both human immunodeficiency virus type 1 Vpr- and gamma irradiation-induced apoptosis. J Virol. 77(3):2063-70.

Yuille MA, Hugunin M, John P, Peer L, Sacks LV, Poiesz BJ, Tomar RH, Silverstone AE. (1988). HIV-1 infection abolishes CD4 biosynthesis but not CD4 mRNA. J Acquir Immune Defic Syndr. 1(2):131-7.

Zagury JF, Cantalloube H, Achour A, Cho YY, Fall L, Lachgar A, Chams V, Astgen A, Biou D, Picard O, et al. (1993). Striking similarities between HIV-1 Env protein and the apoptosis mediating cell surface antigen Fas. Role in the pathogenesis of AIDS. Biomed Pharmacother. 47(8):331-5.

Zauli G, Gibellini D, Secchiero P, Dutartre H, Olive D, Capitani S, Collette Y. (1999). Human immunodeficiency virus type 1 Nef protein sensitizes CD4(+) T lymphoid cells to

apoptosis via functional upregulation of the CD95/CD95 ligand pathway. Blood. 93(3):1000-10.

Zhang S, Pointer D, Singer G, Feng Y, Park K, Zhao LJ. (1998). Direct binding to nucleic acids by Vpr of human immunodeficiency virus type 1. Gene. 212(2):157-66.

Zhou M, Halanski MA, Radonovich MF, Kashanchi F, Peng J, Price DH, Brady JN. (2000). Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. Mol Cell Biol. 20(14):5077-86.

Zhou Y, Ratner L. (2000). Phosphorylation of human immunodeficiency virus type 1 Vpr regulates cell cycle arrest. J Virol. 74(14):6520-7.

Zhu XL, Ayala A, Zellweger R, Morrison MH, Chaudry IH. (1994). Peritoneal macrophages show increased cytokine gene expression following haemorrhagic shock. Immunology. 83(3):378-83.

Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell. 94(4):471-80.