EFFECT OF HIV-1 VIRAL PROTEIN R (VPR) ON T CELL TARGETS:
CONSEQUENCES IN IMMUNOSUPPRESSION AND VIRAL DISSEMINATION

by

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EFFECT OF HIV-1 VIRAL PROTEIN R (VPR) ON T CELL TARGETS: CONSEQUENCES IN IMMUNOSUPPRESSION AND VIRAL DISSEMINATION

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CD4+T-cells have a central role in induction and homeostasis of the immune response, and are also the major target cells for HIV. HIV has devised mechanism(s) to subvert the immune system and further its cause of survival and dissemination. *vpr* is one of the accessory genes, which is essential for the virus survival *in vivo* and has the unique distinction of a non-structural protein incorporated in virus particles in adequate amount, suggesting a role for this protein in the early phase of infection. Being a soluble protein with an ability to transduce across cell membranes, Vpr can potentially affect bystander cells. We hypothesize that HIV-1 Vpr alters the functions of both infected and bystander T lymphocytes, utilizing direct and indirect mechanisms, and these Vpr-mediated effects contribute in part to the immune dysregulation, and aid in viral dissemination. The Specific Aims of this proposal are to: (1) Assess the immune modulatory effects of Vpr in infected and bystander T-lymphocytes; (2) Understand the role of Vpr on T lymphocytes, natural killer (NK) cells and dendritic cells (DC) interactions; and (3) Analyze the structure-function relationship of Vpr in immunopathogenesis. We utilized HIV-1^{wt} and HIV-1ΔVpr viruses and compared the difference in the effects of these viruses under controlled *invitro* conditions. The differences observed in the effect of these two viruses can be attributed to Vpr provided that the infections in both the experimental sets are similar. In some studies, to clearly identify infected cells, we employed EGFP reporter viruses. The effects in infected cells and bystander cells were evaluated. Results indicate that HIV-1 Vpr has a role in

dysregulation of immune cells during HIV infection. Vpr differentially regulates the surface expression of T cell costimulatory molecules, CD28 and CTLA-4, and inhibits IFN-γ production in infected T cells. Vpr also inhibits NK cell function by augmenting TGF-β production and inducing altered expression of NK receptor ligands. Furthermore, oligomerization of Vpr has a role in virion incorporation and in pathogenesis. The findings presented in this study are significant for public health because mechanistic understanding of the pathogenesis will aid in development of novel anti-viral therapeutics.

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1.0   INTRODUCTION

1.1   HIV / AIDS PANDEMIC

Three decades have passed, since acquired immunodeficiency syndrome (AIDS) was first recognized and human immunodeficiency virus -1 (HIV-1) has been identified as the causative agent for this syndrome. The epidemic of HIV/AIDS has spread across the world and is not limited by boundaries of nationality, race, sex or age. It has affected millions of men, women and children across both developing and developed countries. There has been a hundred-fold increase in its prevalence since AIDS was first recognized. The number of AIDS cases gives a foretaste, rather than a true reflection of the health crisis facing the world, for AIDS is the last stage of the infection with HIV that may take many years to cause the syndrome. The HIV/AIDS pandemic continues to expand, with the latest global estimates for people living with HIV/AIDS for the year 2007 standing between 30-36 million, with 3 million people newly affected, the deaths due to HIV infection during this period stand at 2 million. Forty nine percent are men, forty five percent are woman and approximately six percent are children under the age of 15 years [1, 2].

The major route of transmission is heterosexual intercourse, although other routes include intravenous drug abuse, men who have sex with men, mother to child transmission, and transmission through transfusion of blood and blood products. Analysis of epidemiological data across the past decades provides some encouraging trends, reflecting the natural evolution of the

epidemic as well as the effectiveness of preventive and treatment programs. The incidence of HIV infection peaked globally in the late 1990's, and ever since there has been a steady decline in the number of new cases per year. Also the prevalence of global HIV infection has stabilized since the start of 21st century, and a reduction in the number of deaths due to AIDS have also been recorded globally.

1.2   HIV-1 BIOLOGY

1.2.1   HIV Virus Structure - Viral Proteins and Genome

Human immunodeficiency virus (HIV) is a single strand positive sense RNA virus and belongs to genus Lentivirus. Being a retrovirus, HIV has the enzyme reverse transcriptase which helps in conversion of positive sense viral RNA to double stranded proviral DNA. HIV is about 9.7 kilo-basepairs long and encodes for nine viral proteins. They include structural, enzymatic and non-structural proteins. The structural proteins Gag and Env form the bulk of the virus particle. The enzymatic polyprotein Pol is cleaved to reverse transcriptase (RT), integrase (IN) and protease (PR). The nonstructural proteins include regulatory and accessory proteins. The regulatory proteins are Tat and Rev which help to regulate the expression of viral genes. Unlike other members of the retroviridae family, HIV is unique in having the accessory genes, which are vital for in vivo survival of the virus. The accessory proteins include Vpr, Nef, Vpu and Vif. Multiple functions have been attributed to the accessory proteins and they have a positive role in virus life cycle and negatively regulate the host defense mechanisms to favor virus survival in vivo [3].

The viral genes are driven by long terminal repeats (LTR) which flank both ends of the genome. They also have an important role in the process of reverse transcription and in integration of proviral genome [4]. Gag, Group specific antigen, forms the main structural component of the virion particle. Gag is synthesized as a polypeptide, from an unspliced RNA transcript. Proteolytic cleavage of this polyprotein precursor identified by its molecular weight as p55 precursor, gives raise to the smaller gag proteins – p24, p19, p17, p6 that form the capsid (CA), nucleocapsid (NA), and matrix (MA). The nucleocapsid forms the innermost layer of the virus, forming the protective core around the viral genomic RNA [5, 6]. The capsid protein (p24) forms the subsequent layer and is the most abundant viral protein synthesized. Capsid has an important role in modulating the host cell permissiveness to HIV-1 infection through its interaction with host cellular protein - Cyclophilin-A [7]. The matrix protein, p17, is located just below the viral envelope, which contributes to the structure of the virus. It is an important role during virus assembly. Gag p6 has a role in the release of budding virus particles and interacts with the accessory protein, Vpr, and aids in the incorporation of Vpr in virus particles [8].

Pol, polymerase, is also synthesized as a p160 polyprotein, which is cleaved to form three important enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR). Reverse transcriptase is unique to members of the family retroviridae and hepadnaviridae. It helps in conversion of single strand RNA to double strand proviral DNA, which then gets integrated into the host genome by the enzyme Integrase. Viral protease plays a vital role in virus maturation through processing of Gag and Gag-Pol polyproteins. The viral envelope glycoprotein, Env, is also synthesized as a gp160 precursor protein, which is cleaved to form gp120 (SU) and gp41 (TM). They form noncovalent trimers which are important for virus binding, fusion and entry in target cells.

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The regulatory genes, as the name suggests, are involved in regulation of viral gene expression. This is achieved at the transcriptional level by Tat and at the nuclear export of unspliced viral RNA by Rev. Tat enhances the transcription of viral genes from the LTR, in the presence of Tat the RNA polymerase acquires better processivity and has the ability to transcribe the entire HIV genome [9].
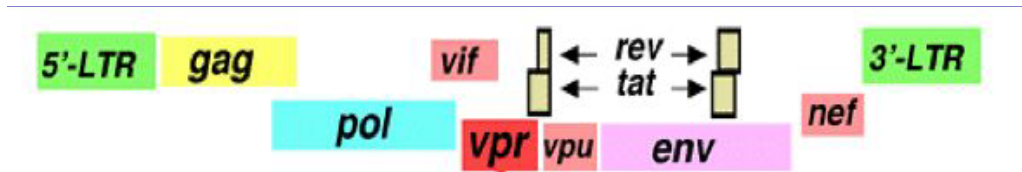
Figure 1. HIV-1 virus genomic organization

Multiple functions have been attributed to accessory proteins, and they are essential for virus survival in vivo [9, 10]. Vpu is known to interact with CD4 at the rough endoplasmic reticulum and degrade it via the ubiquitin-proteosomal pathway. Thus Vpu aids in transport and processing of the envelope glycoprotein gp160, which can potentially be trapped by CD4 in the endoplasmic reticulum, and help in release of virus particles from the producer cells. Vif has a role to increase the infectivity of virus when produced in certain cells. This is due to the ability of Vif to counter the effects of a host restriction factors, APOBEC3G and APOBEC3F, expressed in certain cell types. The accessory protein, Nef, was initially identified as the negative regulatory factor, but over the decades it has been identified to have a diverse role in HIV pathogenesis. Studies have shown that Nef can downregulate critical signaling and regulatory molecules of the immune system. This includes CD4; MHC Class 1 A,B,C; CD28; and β-chain of CD8αβ receptor. Nef also interferes with MHC class II restricted processing of HIV-1 antigen molecules. Additionally, Nef has been recently shown to modulate dendritic cells to kill bystander CD8+T cell by functional up regulation of FasL and TNF-a. Also, Nef induced

inhibition of ASK-1 in HIV-1 infected macrophages suggest that this protein can effectively exploit DC and APC as a transit point on its way to hijack host immune defense [11, 12]. The viral protein R (Vpr), is the focus of this dissertation and has been discussed in detail in subsequent sections. Briefly, Vpr is a pleotrophic protein essential for viral survival in vivo and is incorporated in to Virion particle in significant level [3]. Being a soluble protein and having the ability to transduce across cell membrane, this protein affects both the infected cells and the bystander cells.

1.2.2   HIV-1 Replication Cycle

CD4 is the main receptor of HIV, and the major coreceptors are CCR5 or CXCR4. HIV infects cells, which bear both CD4 and co-receptor. Many other molecules have been identified as receptors and co-receptors [13-17]. Binding of viral envelope to the receptor promotes a conformational change in the envelope that leads to fusion of the virus to the target cell membrane. Following the entry, the viral core is delivered in to the cytoplasm of the cell, and during the process of uncoating there is conversion of viral RNA to proviral DNA by the viral enzyme Reverse transcriptase. Finally the proviral DNA that is part of the pre integration complex (PIC) translocates across the nuclear membrane and can be integrated into the host chromosome. Transcription of the viral genome is driven by the LTR, which is the viral promoter. With the synthesis of viral proteins and export of full-length viral RNA to the cytoplasm, the virions assemble and bud from the plasma membrane.

1.2.3 HIV pathogenesis

HIV was identified as the causative organism for AIDS. Acute HIV infection is symptomatic in a fraction of the individuals infected with the virus and is characterized by a brief flu-like symptoms including low grade fever, sore throat, muscle ache, swollen lymph nodes and rash. These symptoms can present with in to 4 weeks of infection. Following the acute phase, the infection largely remains asymptomatic with low level of virus replication and initiation of a cycle of subtle immunological events, which finally leads to immune deficiency. This chronic asymptomatic phase is highly variable for each individual and can range from 2 years to more than 14 years. The end of this asymptomatic phase is heralded by the onset of AIDS, characterized by low CD4+ T cell counts, high viral loads, increased viral replication fitness, increased genetic variance and a variety of immunological disorders, compromising both the cellular and humoral limbs of immune response, leading to increased susceptibility to opportunistic infections and malignancies [1].
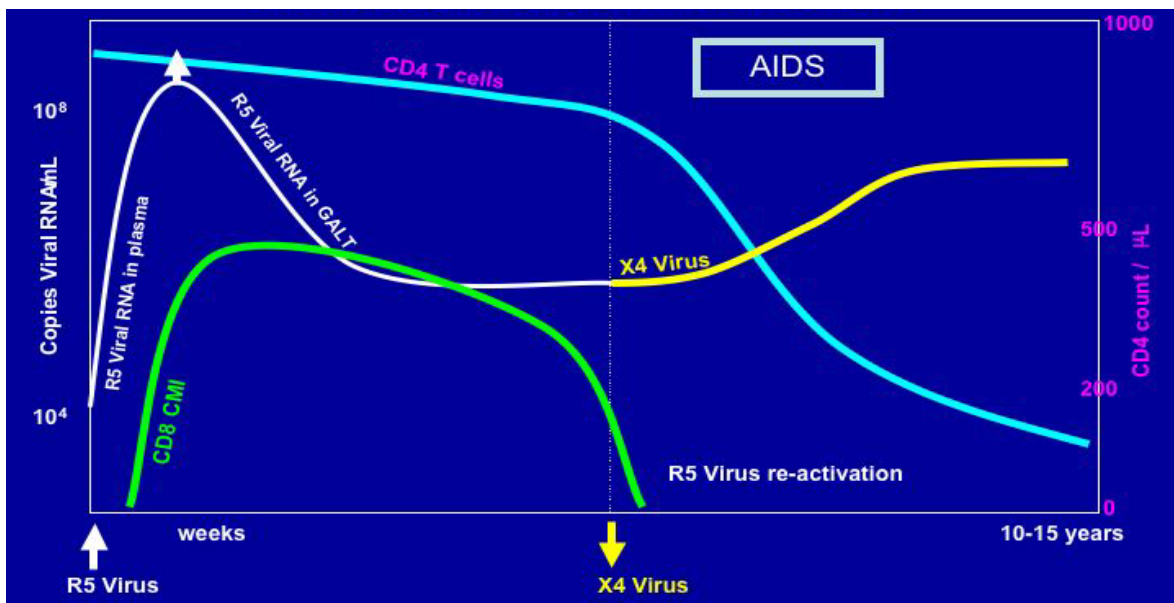
Figure 2. Chronology of untreated HIV infection.

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Upon transmission to a new host, HIV targets cells bearing CD4 surface molecules, which is the main receptor for the virus entry. The major target cells include the CD4+ T cells, macrophages, and dendritic cells. These cells present in the submucosal regions of genital and gastro-intestinal tract are the initial targets following transmission involving mucosal surfaces. There is a massive depletion of these mucosal resident target cells (T cells) within the initial first few weeks of acute infection [18, 19]. Following the mucosal depletion is the state of chronic activation, resulting in increased turnover of activated and memory T cells associated with increased production of proinflammatory cytokines. During this period the CD8+ T cell antiviral response is also high and the ability to control the viremia during the acute phase is usually a good predictor of the rate of progression of infection to AIDS. This is denoted as "viral set point". The lower the viral set point, the slower the rate of progression of infection to AIDS. The virus hijacks the cells of the immune system to facilitate its dissemination from the local tissue. Although a immune response is induced soon after infection, it is inadequate to effectively eliminate the infection.

Seroconversion normally occurs 1 to 3 weeks following infection. During the chronic asymptomatic phase, there is persistent low level virus replication in the lymph nodes and peripheral blood, associated with increased viral genetic diversity and increased viral replicative fitness. The symptomatic phase is characterized by augmented viral load, loss of CD4+ T cells (to less than 200 cells/ul) and escapes from CD8+ T cell response. These events coincide with the increased susceptibility to opportunistic infections and higher incidence of AIDS related malignancies.

1.3   VIRAL PROTEIN R (VPR)

1.3.1   Structure of VPR

The HIV-1 *vpr* gene encodes a protein of 96 amino acids with a predicted molecular weight of 14 kDa, which is conserved in HIV and SIV. Nuclear magnetic resonance (NMR) studies predict that Vpr consists of a three helices connected by turns (Fig. 3). The N-terminal (aa 1–16)
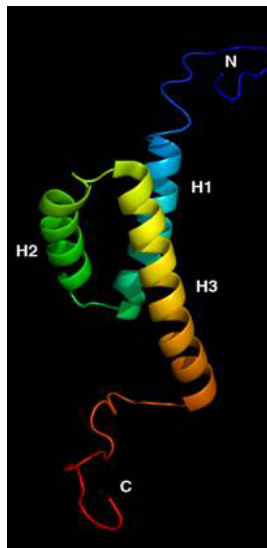
Figure 3. Structure of HIV-1 Vpr.

and C-terminal (aa 78-96) domains are flexible and do not have a definite structure in solution but are critical for Vpr function. The N-terminal domain of Vpr has a role in nuclear localization of Vpr, virion incorporation and interaction with host (RIP1; UNG; Karyopherins; transcription factors Sp1, TFIIB) and viral p6 Gag proteins. The carboxy-terminal region of Vpr plays an important role in cell cycle arrest and also contributes to stability of the protein. Helical domain I (aa 17-33), helical domain II (aa 38–50), and helical domain III (aa 55–77) have essential roles in packing of Vpr into virion particles, stability, nuclear localization and oligomerization [20].

Being a small protein with multiple roles in viral pathogenesis, and essential for in vivo survival of the virus, drastic mutations in any region or domain can potentially alter the structure of the protein. Hence there is a strong selection pressure for conservation of Vpr in the perspective of structure-function. In vitro and in vivo studies have shown that Vpr exists as oligomers. It can form homodimers, trimers and multimers, such an structural organization provides stability and has a critical role in interaction with other host cellular and viral proteins [21-23].

1.3.2   Role of VPR in Viral Pathogenesis

HIV-1 Vpr is incorporated into virus particles through its interaction with the p6 subunit of Gag, making Vpr, as one of the first viral proteins the infected cells get exposed to. Also this interaction with p6 Gag leads to incorporation of the Vpr in both infectious and non-infectious virus particles [24]. Vpr is necessary for efficient infection of non-dividing cells such as macrophages and it enhances viral replication in T cell lines and activated peripheral blood lymphocytes [25]. The presence of extracellular Vpr in plasma and cerebrospinal fluid have been reported and this can result through the following mechanisms: a) synthesis and release of Vpr from infected cells; b) breakdown /lysis of infected cells; and c) breakdown of infectious and non-infectious virus particles, releasing Vpr [26]. Vpr can transduce across cell membranes and can exert its effects on bystander cells which can include even the cells which are not permissible for viral infection [27]. Patients infected with virus encoding defects in the C-terminus of Vpr have slower progression to AIDS [28]. Vpr is a pleiotropic protein. Either as virion associated molecule or extracellular potein, it inhibits cellular proliferation and differentiation. This effect is mediated by modulation of specific host cellular gene transcripts in several cell types [29]. Vpr blocks cell cycling in the G2/M phase of cell cycle, an effect that has been correlated with a

change in the phosphorylation of CDC2 kinases [30]. Vpr can also regulate cellular transcription by suppressing NF-κB activity through effects on IκB synthesis [31]. One particularly intriguing function of Vpr is its ability to mimic the immune suppressor − glucocorticoid, through its interaction with the glucocorticoid receptor (GR) and its response element, GRE [32]. In an *in vivo* model, it has been shown that the presence of Vpr inhibits the induction of the immune response to the co-delivered antigen [33]. Recently Vpr was shown to dysregulate major immune pathways including antigen presentation function, cytokine network and T cell activation by modulating the dendritic cells. These studies suggest that Vpr has a central role in HIV-1 pathogenesis, understanding the details will help in development of novel therapeutics.

1.4   HIV AND IMMUNE SYSTEM

1.4.1   T Lymphocyte costimulatory molecules: Yin–Yang of T cell response

Costimulatory molecules provide the second signal required for optimal activation and differentiation of T lymphocytes upon antigen recognition. CD28 and CTLA-4 are the main co-stimulatory molecules which interact with CD80 and CD86 on the Antigen Presenting Cells (APC) and control the T cell differentiation, proliferation and secretion of chemokines and cytokines [34]. CD28 molecules are constitutively expressed on T lymphocytes, which are up regulated on activation following specific antigen recognition by the T Cell Receptor (TCR). CD28 acts as the positive regulator of T Cell signaling cascade. CTLA-4 is homologous to CD28 and is a counter regulatory molecule, where it is involved along with CD28 in controlling the down stream effects of TCR signaling [35]. CTLA-4 molecules are present in lysosomal vesicles

and are recruited to lipid rafts of the signalosome during immune synapse formation. Signaling through CTLA-4 is related with secretion of immunoregulatory cytokine TGF-β. Also signaling through CTLA-4 is documented to inhibit the CD28 mediated anti HIV-1 effects and facilitate the replication of the virus and also reduce the resistance of neighboring target cells [36]. Dysregulation of CD28 and CTLA-4 on both CD4+ and CD8+ lymphocytes has been well documented in HIV-1 infected patients and is related to progression of the disease [37-39]. Recently it was evaluated that the interaction of costimulatory molecules on DC and T cell triggers signaling in a bidirectional way, both in DC and T cell. In this manner, the T cells can control the signaling in the interacting DC. Interaction of CD28 with the costimulatory molecules, CD80 and CD86 on DC induces the immuno-stimulatory signals in DC whereas, the inhibitory counterpart of CD28 on T cells, CTLA-4 induces tolerogenic signals in DC mediated by IFN-g driven expression of immunosuppressive tryptophan catabolism [40, 41]. Together these studies suggest a role for infected DC-T cell interaction in pathogenesis.

1.4.2   HIV and Natural Killer cells

Compromised NK cell function includes one of the factors for loss of immune competence seen during HIV infection. Studies from our lab and others have demonstrated that the NK cell competence is impaired both invitro and in vivo [42-45]. HIV utilizes several strategies to counter the host NK responses, these include direct effect of viral proteins on NK cells and/or infected cells (example: inhibitory effect of Tat on NK cell functions and differential downregulation of MHC class I molecules on the surface of the infected cells by Nef); and indirect effect through dysregulation of cytokine network leading to compromised NK function. Both the cytolytic and immunomodulatory functions of NK cells are adversely affected during

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the course of HIV infection. There are changes in the NK cell subsets distribution, there is a progressive loss of CD56+ NK cells and accumulation of highly dysfunctional CD56$^-$CD16$^+$ NK cell subset [46].

The presence of defective NK in HIV-1 individuals indicates that HIV-1 infection compromises innate immune response also but the mechanisms involved in this NK cell function dysregulation are not clearly known. Results from our laboratory and others have clearly shown that HIV-1 Vpr has a role in dysregulation of NK cell function in invitro experiments [3, 44-46].

The interaction between NK receptors and their corresponding ligands is critical in determining the ability of NK to discriminate between the normal host cells and virus infected target cells. Both activating and inhibitory set of receptors are present on NK cells. The major activating receptors are mainly the Natural Cytotoxicity Receptors (NCR) NKp30, NKp44 and NKp46 and the C-type lectin, NKG2D. In addition to these activating receptors, another set of NK-cell activating receptors have been identified as coreceptors since they are able to amplify NK cell cytotoxicity when triggered along with the major activating receptor, these include CD244/2B4 receptor and the NK-T-B cell antigen (NTB-A). The inhibitory receptors present on NK (iNKR) have been grouped into three major families, this includes Killer immunoglobulin like receptors (KIR); an heterodimer consisting of CD94 associated with NKG2A/B; and an Immunoglobulin like transcript-2 [45]. These inhibitory receptors interact specifically with different alleles of major histocompatibility complex class I (MHC-I). Though there are difference in the specificity of iNKR, the cytoplasmic regions of all iNKR contain immunoreceptor tyrosine based inhibitory motif (ITIM) which function to recruit and lead to activation of the inhibitory phosphatases, such as Src-homology domain containing tyrosine phosphatases, SHP-1 and SHP-2. Signaling through the inhibitory receptor can overcome and

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modulate the signal through the activating receptor thus, the final outcome depends on the strength of signal input from various activating and inhibitory receptors. Also alterations in ligands for NK cell receptors on target cells have been reported to regulate the corresponding NK cell receptor and their function. Further the strength and density of interaction is critical in determining the calcium mobilization and association with adaptor molecules involved in signaling. Pathogens including HIV are known to modulate both the receptors on NK and the ligands present on the target cells. HIV-1 infection is characterized by a dramatic increase in inhibitory receptors and loss of activating receptors especially NKp30 on NK cells, resulting in loss of NK cell activity. More recently genetic link has been established with certain NKR (e.g. KIRDS3 and its ligand Bw4) repertoire and HIV-1 disease progression. Results also indicate that the ligands for NK receptor are differentially modulated in infected cultures. Modulation of expression of MHC class I molecules during HIV-1 infection is well studied. HIV-1 viral protein Nef is known to specifically down regulate MHC class I A and B, while it has no effect on MHC class I C and E. Further there is loss of activating ligands on the infected cells. These help the virus infected cells to be protected from NK cell lysis. Recently it was reported that HIV-1 Nef has a role in down regulation of NK cell activating ligand MIC A/B on infected cells, this may further aid the infected cells to be protected from NK cells.

1.4.3   T cell–Dendritic cell Cross talk during HIV infection

Dendritic cells (DC) are one of the major professional antigen presenting cells (APC). Interaction of T cell and DC are essential for eliciting an effective immune response. The antigen presented along with the MHC molecule on the DC, is recognized by specific T cell Receptor (TCR). CD28 and CD40L on the T cell interact with CD80/CD86 and CD40 respectively, present on

immature DC, and increase the surface expression of CD80, CD86 and CD83, there is also an increase in the surface expression of CD40 on DC. All these process result in maturation of DC, which has a better ability to present antigens.

DC are one the initial targets of HIV-1 virus. Both X4 and R5 virus can replicate in DC [47]. Reports suggest that infection and replication of HIV-1 virus in the infected cells are facilitated by cell-to-cell contact between DC and T lymphocytes, but the underlying mechanisms are not clear. Further, the replication of HIV-1 in T cell-DC conjugates is more robust than in either cells alone [48]. The transfer of virus from DC to T cell has been well studied, this can be the consequence of trans infection in DC, where the DC concentrate the virus and deliver it to the interacting T cell at the virological synapse, where receptors for HIV-1 on the T cells are recruited [49]. Cis infection of DC results in prolonged transmission of the virus to T lymphocytes [50]. Also DC can pack virus in exosomes and deliver it to the T cells to infect them in an envelope dependent way [51].

Interaction of DC and lymphocytes can occur at different sites, it can be the site of primary entry of the virus or a lymphoid aggregation in the submucosal layer or the secondary lymphnode. Analysis of biopsies from asymptomatic HIV-1 seropositive patients showed high p24 positive cells which expressed markers of both DC and lymphocytes[52]. Further, robust replication of HIV occurs in Peyer's patches, through all stages of the disease and has been implicated in the loss of CD4+ T Lymphocytes [18].

In addition to the ability of virus to exploit the DC functions to facilitate its spread, it also impairs the ability of DC to induce an effective immune response. HIV viral proteins are known to have varied effect on the DC. The outcome depends on the protein and the DC cell type and the invitro conditions, the DC are maintained in. HIV accessory proteins Vpr and Nef impair the

ability of DC to upregulate co-stimulatory molecules following second signal and disrupt the normal cytokine secretion profile, skewing towards an immunosuppressive profile. Increased TNF-α and other related death molecules on the DC contributes to apoptosis of bystander CD8+ T cells. Understanding the DC-T cell interaction and elucidating the viral mechanisms / factors which subvert the physiologically critical interaction, essential for induction of an effective immune response, to a virological synapse, which is important for efficient virus dissemination, will help us develop novel therapeutics and newer anti-viral strategies.

2.0   HYPOTHESIS AND SPECIFIC AIMS

2.1   RATIONALE

HIV-1 infection leads to chronic disease, with the Acquired Immunodeficiency Syndrome developing decades after infection. Even though, loss of CD4+ T cells is the hallmark of onset of AIDS, the process is a gradual one involving a cycle of immunological events preceding the loss of T cells, where the target cells are dysregulated. CD4+ T cells are the main target cells for HIV, and they have a central role in homeostasis of immune response. HIV has devised methods to achieve its objective of survival, by utilizing the CD4+ T cells to suppress the immune response and favor the dissemination of virus.

HIV-1 Vpr is one of the accessory genes which is essential for the virus survival *in vivo* and is incorporated in virus particles in adequate amount, suggesting a role for this protein in the early phase of infection. Vpr also has a central role in modulating basic cellular functions including cellular differentiation, cell cycle and apoptosis. Being a soluble protein with an ability to transduce across cell membrane, Vpr can potentially affect bystander cells. Studies have underlined the role of Vpr to modulate immune response, including critical cell surface molecules, basic signaling pathways and cytokine networks.

2.2   HYPOTHESIS

I hypothezise that, HIV-1 Vpr alters the functions of both infected and bystander T lymphocytes, utilizing direct and indirect mechanisms, and these Vpr-mediated effects contribute inpart for the immune dysregulation and aid in viral dissemination.

The Specific aims of this proposal are:

1. Assess the immune modulatory effects of HIV-1 Vpr in infected and bystander T-lymphocytes *in vitro*.

2. Understand the role of HIV-1 Vpr in T lymphocytes, natural killer (NK) cells and dendritic cells (DC) interactions.

3. Structure-function analysis of HIV-1 Vpr in immunopathogenesis, virus replication and disease progression: Focus on Vpr oligomerization and functions.

3.0 IMMUNE MODULATORY EFFECTS OF HIV-1 VPR IN INFECTED AND BYSTANDER T-LYMPHOCYTES *IN VITRO*: DIRECT EFFECT ON T CELL ACTIVATION, SURVIVAL AND IMMUNE FUNCTION.

This chapter includes results that were published in "Venkatachari, N.J., B. Majumder, and V. Ayyavoo, *Human immunodeficiency virus (HIV) type 1 Vpr induces differential regulation of T cell costimulatory molecules: direct effect of Vpr on T cell activation and immune function.* Virology, 2007. **358**(2): p. 347-56."

3.1   INTRODUCTION

Costimulatory molecules expressed in T cells provide the second signal required for optimum activation and differentiation of T lymphocytes upon antigen recognition [53, 54]. They have a role in regulation of immune response and survival of the cells. Most of these processes are controlled by positive and negative signals transduced across cell membrane following the interaction of cell surface molecules with their ligands. CD28 and CTLA-4 are the main costimulatory molecules in T cells that interact with CD80 and CD86 on the antigen presenting cells (APC) and initiate proliferation, differentiation and effector functions [34, 55, 56]. Expression of CD28 molecules increases following specific antigen recognition through TCR and acts as the positive regulator of the T cell signaling cascade. In contrast, CTLA-4, a CD28 homolog, negatively regulates the effects of TCR signaling [57, 58]. The effectiveness of the immune response, including the secretion of cytokines, is controlled by the interaction of CD28 and CTLA-4 with their ligands on the APC.

Therefore, differential expression of these important molecules can skew the outcome of the immune response. Dysregulation of CD28 and CTLA-4 in T lymphocytes has been well documented in HIV-1 infected patients and is correlated with disease progression [36-39]. Additionally, signaling through CTLA-4 is documented to counteract CD28 mediated anti-HIV-1 effects and facilitate the replication of the virus and enhance the susceptibility of neighboring cells [36].

HIV-1 exploit various mechanisms to modulate the function of important cellular molecules in T lymphocytes at multiple levels. HIV-1 Nef protein is involved in accelerating the endocytosis of CD4, CD28 and MHC class I molecules [59-61], whereas the *vpu* gene product has been reported to affect the biosynthesis of MHC class I molecules and cause ubiquitination-dependent degradation of CD4 molecules in infected T-Lymphocytes [62, 63]. The ability of Env, Nef and Vpu to disrupt the expression of CD4 molecules in infected T lymphocytes collectively suggests that HIV-1 viral proteins disrupt the normal host cellular immune pathways in T lymphocytes thus exploiting the cellular machinery for viral replication and survival [64-67].

HIV-1 *vpr* gene encodes a 96 amino acid protein that is incorporated into the virus particle through its interaction with p6 subunit of Gag, thus making Vpr as one of the first viral proteins that the infected cells are exposed to, prior to *de novo* synthesis [20, 68, 69]. Patients infected with HIV-1 encoding defects in Vpr have slower progression to AIDS [70-72]. Vpr was shown to dysregulate major immune pathways including antigen presentation, cytokine network and T cell activation, by impairing dendritic cell function as antigen presenting cells (APC) [73, 74]. However, the direct effect of Vpr in infected T cells and its immune functions are not clearly understood. Using enhanced green florescence protein (EGFP) coding reporter viruses, with and without *vpr*, here we evaluated the effect of HIV-1 Vpr on T cell costimulatory molecules, CD28 and CTLA-4, as well as the functional consequences of Vpr expression in T cell activation and survival in both infected and bystander cells. Results indicate that HIV-1 Vpr differentially regulated the expression of cell surface molecules and impaired IFN-γ production that is involved in T cell activation functions. Additionally, HIV-1 Vpr inhibited the nuclear translocation of NF-κB in infected T cells. Together, these results suggest that Vpr can

selectively alter various immune regulatory molecules at multiple levels in infected T lymphocytes to escape host immune response.

3.2   MATERIALS AND METHODS

3.2.1   Isolation and culture of Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from healthy donors using Ficoll-Hypaque gradient centrifugation. CD14 depleted lymphocytes were purified by negative selection using anti-CD14 monoclonal antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as described previously [74]. Briefly, PBMC were resuspended in MACS buffer (2 mM EDTA, 0.05% bovine serum albumin in phosphate-buffered saline [PBS]) and incubated with anti-CD14 MACS beads at 4 °C for 15 min. Cells were washed with MACS buffer and resuspended in MACS buffer before passing through a magnetic column. The flow through was collected, and the purity of the lymphocytes was tested by flow cytometry using CD14-phycoerythrin (PE) (clone − M5E2, BD-Pharmingen, San Diego, CA) and CD3-ECD (Beckman Coulter, Miami, FL). More than 95% of isolated cells were CD14$^-$ (data not shown). PBL (1×10^7/ml) were stimulated with anti-CD3 (OKT3) antibody (10 μg/ml)-coated flasks along with soluble anti-CD28 antibody (BD Pharmingen clone 28.2) (1 μg/ml) for 3 days as described [75] and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin and rIL-2 (5 U/ml).

3.2.2 Construction of HIV-1wt and HIV-1Δvpr EGFP reporter proviral plasmids

To evaluate the effects of Vpr in productively infected cells, we constructed EGFP reporter viruses, expressing full-length Vpr protein (HIV-1 vpr(+)/EGFP) and the other which does not express Vpr (HIV-1 vpr(−)/EGFP). An Internal Ribosomal Entry Site (IRES) site derived from ECMV virus was inserted to maintain the expression of Nef (Figure 5A). The *vpr* gene open reading frame in HIV-1 vpr(−)/EGFP virus has mutation at nucleotide 56, resulting in no detectable Vpr expression as shown by Western blot. The integrity of the viral DNA sequence was confirmed by sequencing, and the expression of viral proteins Gag, Vpr and Nef were confirmed by Western blot and/or by functional assays.

3.2.3 Virus preparation and infection of T cells

HEK293 T cells (2×10^6 per plate) were transfected with 10 μg of HIV-1 vpr(+)/EGFP or HIV-1 vpr(−)/EGFP proviral construct by calcium phosphate precipitation method [74]. Forty-eight hours posttransfection, the supernatants were collected, filtered through a 0.4-μm filter to remove cellular debris, and centrifuged at 22,000 rpm for 1 h. The virus pellets were resuspended in PBS and stored in aliquots at − 80 °C for subsequent assays. Virus titers were measured by p24 enzyme-linked immunosorbent assay (ELISA), and multiplicity of infection (MOI) was calculated by TZM blue assay using the HIV-1 reporter cell line cMAGI (AIDS Research and Reference Reagent Program [RRRP], National Institutes of Health [NIH]). PBL were infected with the HIV-1 vpr(+)/EGFP or HIV-1 vpr(−)/EGFP reporter virus at a MOI of 0.01. Six hours post infection, the viruses were removed by washing, and cells were maintained in R10 media containing rIL-2 (5 U/ml).

3.2.4 Flow Cytometry

To confirm the purity of lymphocytes, we tested the phenotype of the cells by flow cytometry. Cells were stained with CD14-PE and CD3-ECD or with a corresponding fluorochrome-conjugated IgG isotype control. Expression of costimulatory and other surface markers were evaluated by flow cytometry. Surface staining was performed for CD28, CD25, HLA-DR and MHC class I, whereas for CTLA-4, both surface and intracellular staining was performed. Briefly, at indicated time points cells were washed twice with cold PBS (pH 7.2) containing 10% FBS and incubated with respective fluorochrome conjugated antibody or isotype control for 1 h at 4 °C. The cells were washed three times with fluorescence-activated cell sorter (FACS) buffer. For the detection of intracellular proteins, fixation and permeabilization were carried out using the CytoFix-CytoPerm kit (BD-Bioscience, Mountainview, CA). The cells were fixed in the BD CytoFix-Cytoperm solution for 20 min, followed by two washes with Perm-Wash buffer (BD-Bioscience). Intracellular staining was performed at room temperature for 1 h using the recommended dilution of antibody, per 10^6 cells, followed by two washes in Perm-Wash buffer and analyzed by flow cytometry. Gating was done as described below. Firstly, live lymphocytes were gated based on Side scatter and Forward scatter. Based on this, CD3+ cells were gated to eliminate the CD3− population (NK cells), and then infected and uninfected (bystander) cells were identified based on the expression of EGFP within the CD3+ population. Intracellular cytokine staining was carried out following the standard staining protocol. Cells (1×10^6) at the mentioned time point were stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (2 μl/ml) for a duration of 8 h, followed by incubation at 4 °C for 6 h. Intracellular staining was done with anti-IFN-γ or IgG1 isotype control using the same protocol as described above. Samples were analyzed using Epics-XL (Beckman Coulter, Miami, FL) with

minimum of 20,000 gated events acquired for each sample, and the mean fluorescence intensity (MFI) was calculated using Cell Quest and Flow Jo software.

3.2.5 Western blotting

Lymphocytes were cultured and infected as described above for 3 days. A total of 5×10^6 uninfected and infected lymphocytes were washed twice with PBS and lysed in RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1.0 mM phenylmethylsulfonyl fluoride, 0.05% deoxycholate, 10% sodium dodecyl sulfate, aprotinin (0.07 trypsin inhibitor unit/ml), and the protease inhibitors leupeptin, chymostatin, and pepstatin (1 μg/ml; Sigma). Cell lysates were clarified by centrifugation, and total cell lysates (50 μg) were separated on a 10.5 to 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis gel, transferred, and immunoblotted with anti-HIV-1 p24, anti-HIV-1 Nef (AIDS RRRP, NIH), and anti-HIV-1 Vpr (a kind gift from John Kappes, University of Alabama) antibodies. Tubulin was used as loading control. The blots were developed using an ECL kit (Amersham Biosciences, Piscataway, NJ).

3.2.6 Immunofluorescence

Forty-eight hours post infection, lymphocytes were washed with PBS, and attached onto slides using cytospin. Cells were fixed using in 3.7% formaldehyde at room temperature for 10 min, washed and permeabilized with 0.5% Triton X-100 for an additional 10 min. After washing 3 times with PBS, cells were blocked with 5% BSA at room temperature for 1 h followed by incubation with primary antibody (NF-κB (p65) (1:200 dilution, Santa Cruz)) for 1 h at room

temperature. Cells were washed 3 times with 1× PBS, and incubated with goat anti-rabbit IgG Rhodamine (RRX) (1:400; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Following three washes with 1× PBS, cells were mounted with VECTASHIELD mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence was detected using a fluorescence microscope with Nikon SPOT camera (Fryer, Huntley, IL) and images were processed using MetaMorph software (Universal Imaging Corporation, Downington, PA).

3.2.7 IFN-γ ELISA

Following infection of PBL with HIV-1 vpr(+)/EGFP or HIV-1 vpr(−)/EGFP, supernatants were collected and analyzed for the presence of IFN-γ. INF-γ was measured by using Opti-EIA enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA) according to the manufacturer's protocol.

3.2.8 Statistical analysis

The results were expressed as mean ± standard error of the mean. The data were analyzed using the Student's *t* test for paired samples.

3.3   RESULTS

3.3.1   Construction and characterization of HIV-1 vpr(+) and HIV-1 vpr(-) EGFP reporter viruses

To clearly distinguish the role of HIV-1 Vpr on infected and uninfected T cell immune functions, and to further understand the contribution of Vpr in immunopathogenesis, EGFP reporter viruses were constructed. This was achieved by the insertion of EGFP reporter and IRES fragment in frame with the nef open reading frame, which maintained the Nef expression (Fig.4A). To determine the expression of viral proteins, PBL were infected as described in Materials and methods. Three days post infection cells were lysed, and lysates were analyzed for expression of Gag (p24), Nef and Vpr by immunoblot (Fig.4B). Immunoblot analysis indicates the expression of viral proteins confirming the integrity of these constructs. Additionally, function of Nef was evaluated by assessing the ability of Nef to downregulate MHC I molecules in the infected T cells. Viruses generated from 293T cells were used to infect Jurkat JJK cells and the expression of MHC I and CD3 was tested by flow cytometry (Fig.4C). As a control, pNL43 Δ Nef/EGFP (kind gift from Dr. David N. Levy, University of Alabama) was used. Results indicate that both the HIV-1 vpr(+)/EGFP and HIV-1 vpr(-)/EGFP constructs downregulated the expression of MHC I in the infected cells (> 40%), compared to the HIV-1 Δ Nef/EGFP virus (< 2%), indicating that both the expression and function of Nef were maintained in these constructs. EGFP expression was also evaluated by immunofluorescence microscopy, and the ability of this reporter virus to distinctly identify the infect cells was confirmed by flow cytometry.

Next, to confirm that the insertion of the IRES-EGFP fragment into the NL43 viral genome did not impair its replication competence, anti-CD3/anti-CD28-stimulated normal human PBL were infected, and virus replication was monitored. Both viruses infected PBL efficiently and supported virus replication, as measured by p24 production (Fig.4D). However, in the presence of Vpr, there was a slight increase in the p24 levels in the HIV-1 vpr(+)/EGFP virus, compared to the Vpr-negative counterpart, as observed before [76, 77]. Together these results indicate that insertion of EGFP-IRES fragment did not alter either the expression or the function of Nef, further confirming the specific effects of Vpr in the presence of other viral proteins.
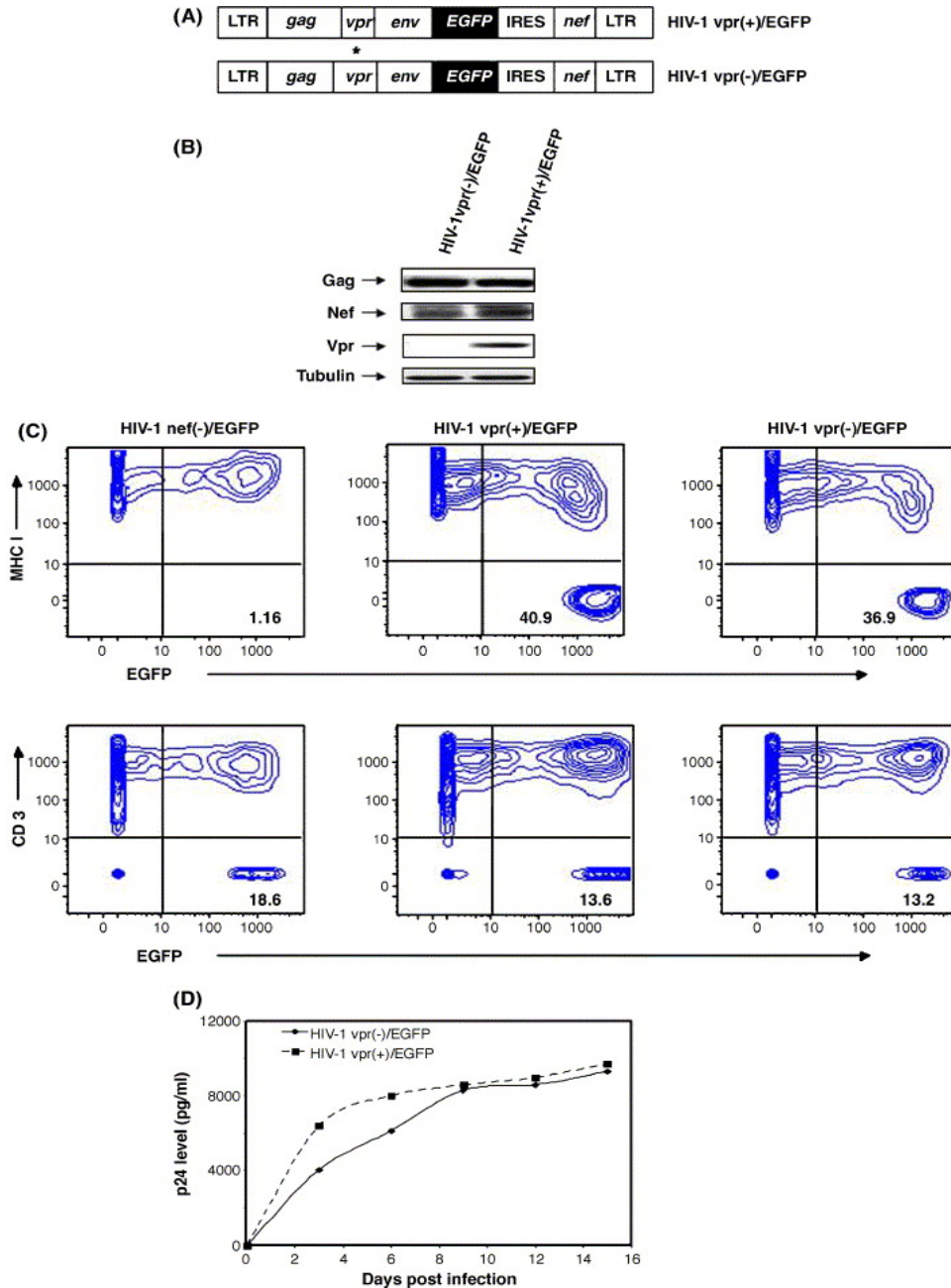
Figure 4. Construction and characterization of EGFP-containing HIV-1 vpr(+) and HIV-1 vpr(−) virus.

(A) Schematic representation of viral constructs. EGFP reporter virus HIV-1 vpr(+)/EGFP and HIV-1 vpr(-)/EGFP, showing the site of insertion of the IRES sequence in the HIV-1 viral genome to maintain the expression of both EGFP and Nef. (B) Expression of viral proteins by immunoblot: Cell lysates obtained from infected PBL on day 6 post infection with the EGFP reporter virus HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP were characterized for the presence of Gag, Vpr and Nef by immunoblot analysis using specific antibodies. Tubulin was used as loading control. (C) Functional analysis of nef in the reporter virus: Jurkat JJK cells were infected with the reporter virus HIV-1 Nef(-)/EGFP, HIV-1 vpr(+)/EGFP and HIV-1 vpr(-)/EGFP. Five day post infection the expression of MHC class I and CD3 were evaluated on the surface of infected cells by flow cytometry. (D) Replication kinetics of the vpr(+) and vpr(-) reporter virus in PBL: Cells were infected with the EGFP reporter virus HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP as described in Material and Methods. At the indicated time points the supernatant was collected and p24 was estimated by ELISA.

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3.3.2 HIV-1 Vpr differentially regulates the expression of costimulatory molecules CD28 and CTLA-4 in T cells

To determine the effect of HIV-1 Vpr costimulatory molecules CD28 and CTLA-4 in vitro and its functional impact on host immune responses, monocyte-depleted PBL (> 95% as measured by flow cytometry) were activated with anti-CD3 and anti-CD28 antibody and infected with 0.01 MOI of HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP virus. Four days post infection, expressions of CD28, CTLA-4, CD25, and HLA-DR were evaluated by flow cytometry. To distinguish the Vpr-mediated effects in infected and bystander cells, cells were first gated based on forward and side scatter, followed by gating on CD3-positive cells. Within the CD3-positive cells, infected and bystander cells were separated based on EGFP positivity. Results indicate that there is no significant difference in the number of cells (shown in percent positive) infected with either HIV-1 vpr(+) or HIV-1 vpr(-) virus (Fig.5A). Using this gating, expression of CD28 was assessed in both EGFP-positive and EGFP-negative cells. In HIV-1 vpr(+)-infected cells (considering the EGFP-positive as 100%), the number of cells expressing CD28 was significantly reduced (67.2%) compared to the HIV-1 vpr(-)-virus-infected cells (94.2%), whereas no significant change was observed in the bystander/uninfected cells (92.1 versus 94.5; Fig.5B). Additionally the level of expression of CD28, as measured by mean fluorescence intensity (MFI), was also lower in the HIV-1 vpr(+)-virus-infected cells (143) compared to the HIV-1 vpr(-)-virus-infected cells (239). These studies were performed in PBL isolated from multiple donors (N = 7) and similar reduction was observed (Fig.5C) indicating that HIV-1 Vpr significantly (p = 0.0014) downregulated one of the important costimulatory molecules, CD28 in infected cells.

The significant reduction in CD28 expression in infected T cells, caused by HIV-1 Vpr prompted us to measure the effect of Vpr on the expression of its counter regulator molecule CTLA-4. Unlike the constitutively expressed CD28, CTLA-4 is expressed only upon activation and mainly resides in intracytoplasmic vesicles, and then recruited to the immunological synapse to regulate signal transduction at the proximal level [78]. To evaluate the effect of Vpr on the expression of CTLA-4, both cell surface and intracellular CTLA-4 were stained and assessed in PBL from the same donor by flow cytometry using similar gating as shown in Fig.5A. Fig.5D indicates that presence of Vpr increased expression of CTLA-4 in the infected cells. Cells expressing CTLA-4 are 30.6% in HIV-1 vpr(+)/EGFP-virus-infected culture, whereas, only 18% of HIV-1 vpr(-)/EGFP-virus-infected cells expressed the inhibitory costimulatory molecule, CTLA-4. Similar results were observed in multiple donors (N = 7), as shown in Fig.5E (p = 0.026), further indicating that the observed increase in CTLA-4 expression is Vpr specific and not donor-dependent.
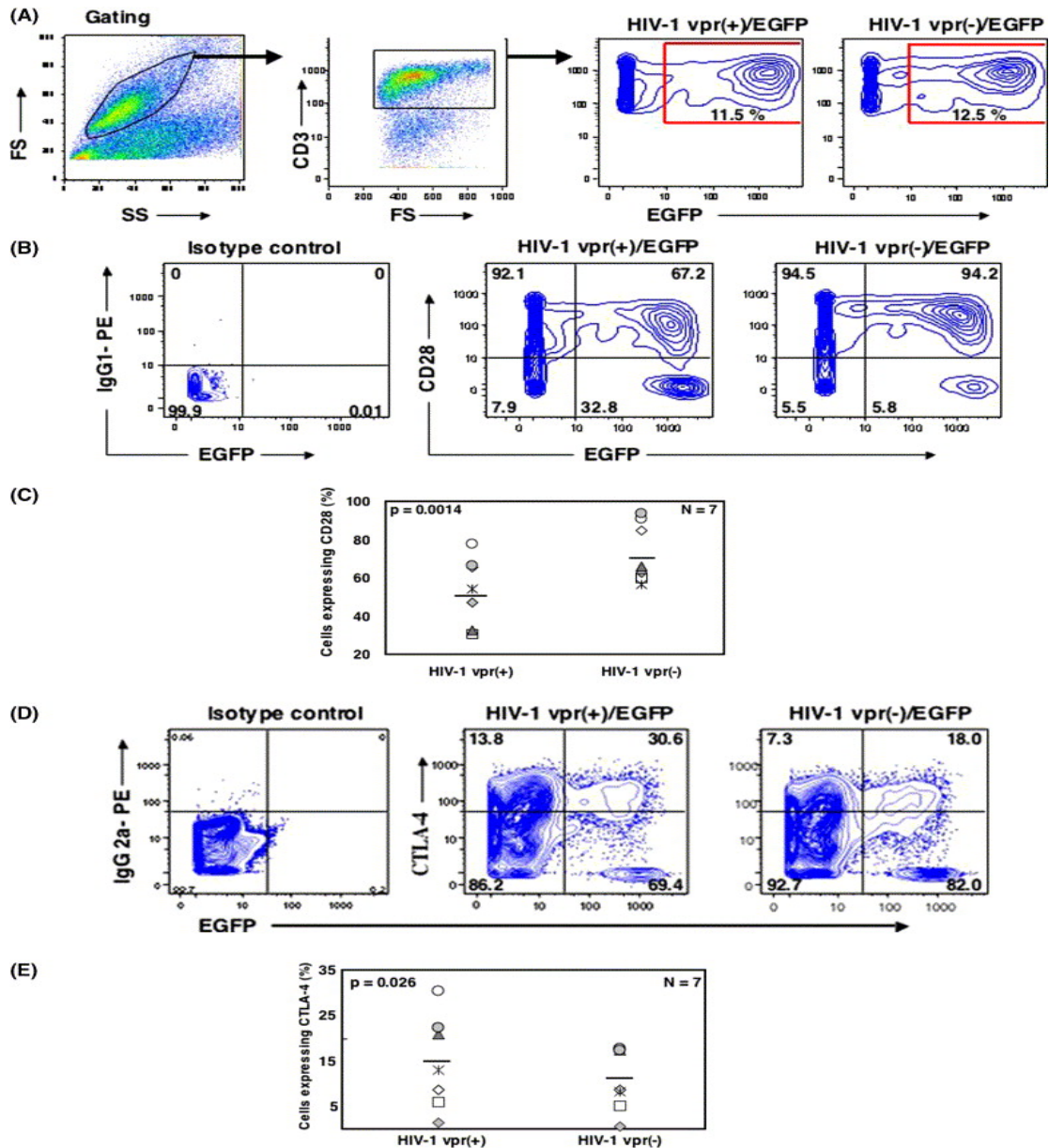
Figure 5. Differential regulation of T cell costimulatory molecules by HIV-1 Vpr.

(A) PBL were infected with HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP as described. Four days post infection the percentage of infected cells was analyzed by flow cytometry. Viable lymphocytes were gated based on side and forward scatter dot plot. The CD3 expressing cells were then gated, and the Infected (EGFP+) and Bystander (EGFP-) cells were identified based on EGFP expression. (B) Expression of CD28 was analyzed by flow cytometry using directly conjugated specific antibodies and isotype controls. The cells were gated as described in Panel A, and the expression of CD28 was analyzed on the infected (EGFP+) and bystander (EGFP-) cells. Each population (EGFP+ or EGFP-) was considered as 100% during analysis. (C) Downregulation of CD28 by Vpr in multiple donors (N=7). p value was derived using paired t test analysis comparing HIV-1 vpr(+) and HIV-1 vpr(-) groups. (D) Expression of CTLA-4 was determined by flow cytometry. Cells were stained for surface and intracellular expression of CTLA-4 as described in Materials and Methods. (E) HIV-1 Vpr differentially regulates the expression of CTLA-4 in multiple donors (N=7). p value was evaluated by paired t test analysis.

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Next, to rule out the possibility that HIV-1 Vpr did not alter the expression of T cell receptors globally, surface expression of CD25 and HLA-DR molecules was measured within the infected and uninfected population using the same donor cells (Fig.6A). There is no significant change in the surface expression of CD25 and HLA-DR molecules in the presence or absence of Vpr, in both the infected as well as the bystander cells. Similar results were observed in multiple donors (Fig.6B). These results indicate that the dysregulation of CD28 and CTLA-4 shown above is specifically regulated by the *vpr* gene product. A decrease in CD28, the positive regulator of the signal transduction, combined with an increase in negative regulator, CTLA-4 can have an additive effect to impair proximal signal transduction following the recognition of specific antigen by the T cell receptor [79, 80].
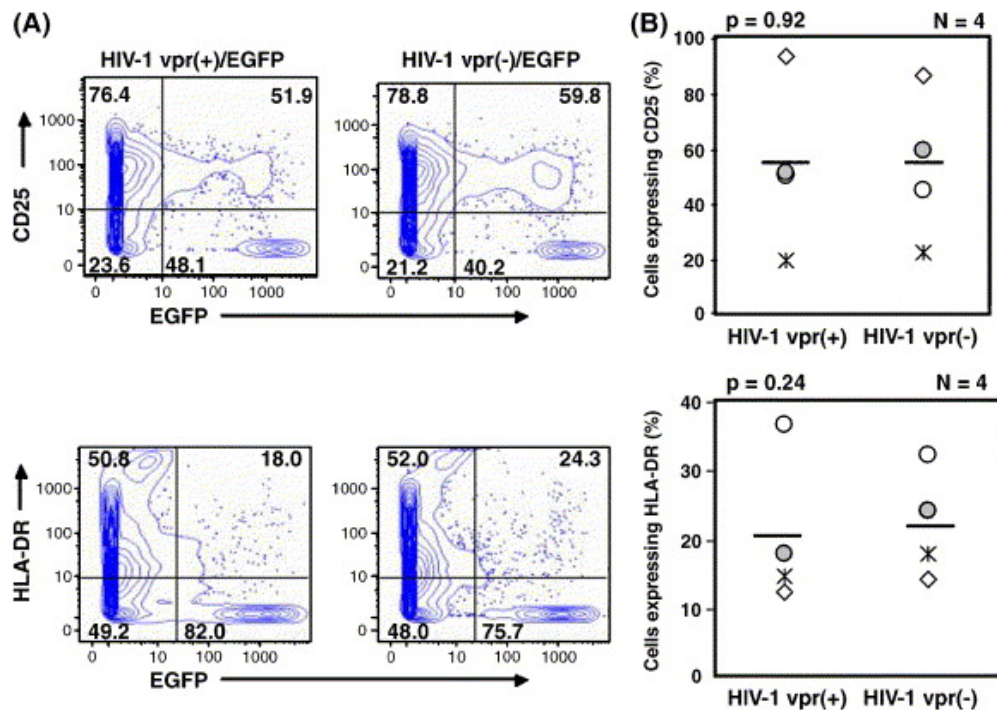
Figure 6. Effect of HIV-1 Vpr on CD25 and HLA-DR in infected and bystander cells.

PBL were infected with EGFP reporter viruses as described in Material and Methods. Four days post infection, the cell surface expression of CD25, and HLA-DR was analyzed by flow cytometry. Viable lymphocytes were gated as shown in Fig.6A. Expression of CD25 and HLA-DR was analyzed in infected (EGFP+) and bystander (EGFP-) cells. (A) Representative contour plot for one of the multiple donors. (B) Dot plot represents data from four donors.

3.3.3 HIV-1 Vpr inhibits interferon gamma (IFN-γ) secretion in infected T cells

Interferon gamma is one of the key Th1 cytokines secreted by T lymphocytes upon activation and is required for antiviral immunity. It has been well documented that there is a decline in the IFN-γ secreting cell population during HIV-1 infection, and this correlates with the compromised T-cell-mediated response patients [81-83]. Hence we evaluated the amount of IFN-γ secreted by PBL infected with HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP virus by ELISA on days 3 and 6 post infection to further delineate the potential implications of Vpr. Results indicate that in the presence of Vpr there is a significant decrease (> 50%) in IFN-γ production compared to Vpr-negative virus-infected culture on day 6 post infection (Fig.7A). These results were consistent in multiple donors (N=4), as depicted in Fig.7B. To further quantitate the level of IFN-γ production within the infected cells, intracellular staining for IFN-γ was performed. Number of infected cells that are positive for intracellular IFN-γ was threefold lower in HIV-1 vpr(+)/EGFP-virus-infected culture compared to that in HIV-1 vpr(-)/EGFP-virus-infected culture. In case of HIV-1 vpr(+)/EGFP-infected cells, only 3.55% of cells were positive for IFN-γ, whereas 11.8% of HIV-1 vpr(-)/EGFP-infected cells produced IFN-γ (Fig.7C). These data suggest that HIV-1 Vpr can mediate its inhibitory effects in the presence of other viral proteins.
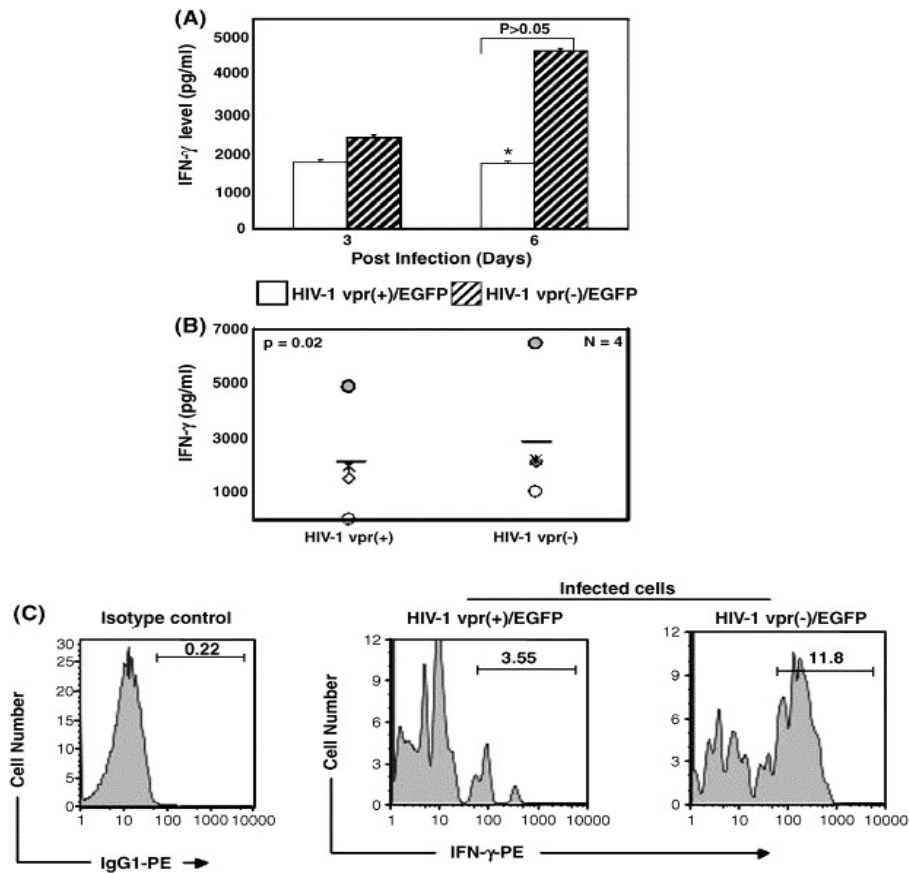
Figure 7. HIV-1 Vpr inhibits IFN-γ production in HIV-1-infected lymphocytes.

(*A*) *PBL were infected with the EGFP reporter virus HIV-1 vpr(+)/EGFP or HIV-1 vpr(-)/EGFP as described in Material and Methods. The supernatant collected 3 and 6 day post infection was assessed for IFN-g production by ELISA. The error bars denote the standard error of the mean, obtained from samples in triplicates. (B) Production of IFN-g in multiple donors (N=4) was evaluated statistically evaluated by paired t test. (C) Detection of IFN-g by intracellular staining using PE conjugated IgG1 isotype or anti-human IFN-g antibody following fixation and permeabilization, using flow cytometry. Viable lymphocytes were gated and IFN-g production with in the infected (EGFP+) and bystander (EGFP-) lymphocytes was evaluated. The histograms represents one of the multiple donors (N=4) evaluated.*

3.3.4   HIV-1 Vpr inhibits the nuclear translocation of the p65 member of the NF-κB family in infected cells

Results presented above indicate that HIV-1 Vpr inhibited the production of IFN-γ in HIV-1-infected cells, suggesting that HIV-1 Vpr not only selectively modulates the cell surface

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expression of the major costimulatory molecules CD28 and CTLA-4, but also interferes with downstream events of T cell activation. NF-κB is one of the important cellular factors that regulate the production of IFN-γ upon activation. Therefore, we further evaluated the effect of HIV-1 Vpr on NF-κB translocation in infected lymphocytes by immunofluorescence to elucidate the involvement of Vpr in the context of infection. Results indicate that in more than 70% of HIV-1 vpr(+)/EGFP-virus-infected lymphocytes NF-κB is localized in the cytoplasm, whereas in HIV-1 vpr(-)/EGFP-virus-infected cells, NF-κB is located predominantly in the nucleus (Fig.8A). A similar pattern was seen in multiple donors (N = 4) as shown in Fig.8B. Though previous studies have suggested that endogenous expression of Vpr in the absence of other viral proteins inhibits the transcriptional regulation of NF-κB in cell lines [31], this is the first report indicating a similar phenomenon in HIV-1 infected primary lymphocytes. Together these results indicate that one of the mechanism(s) exerted by Vpr is to block NF-κB mediated transcriptional regulation as part of the immune evasive strategies as noted in other viruses [84, 85].
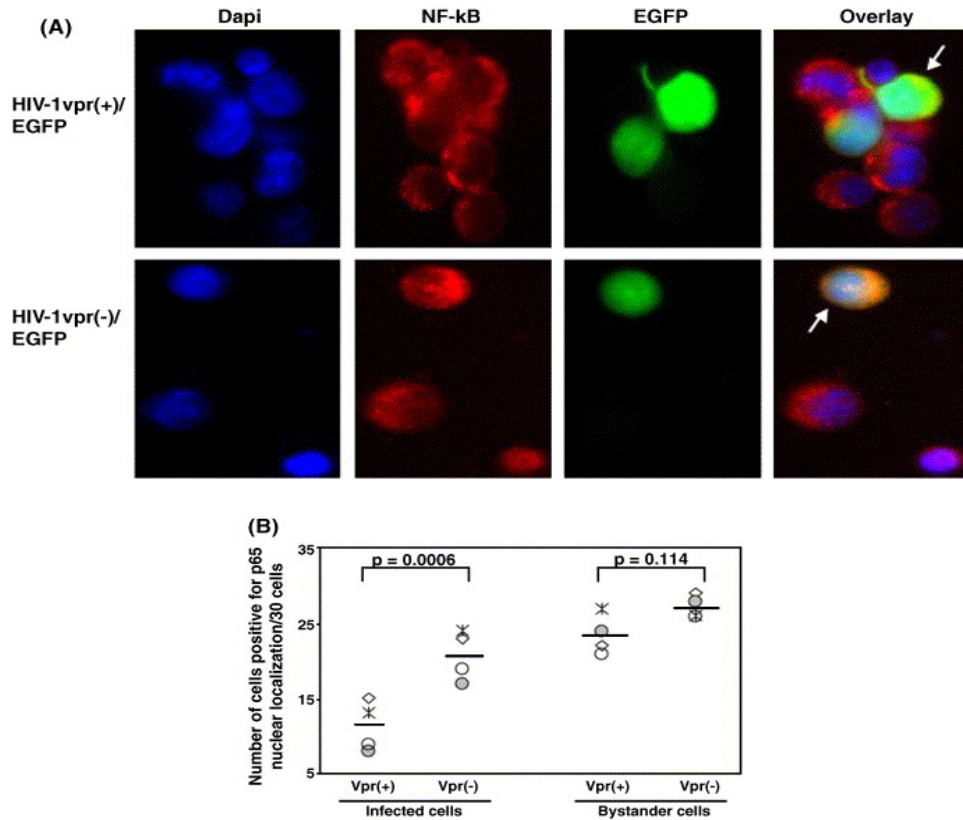
Figure 8. Subcellular localization of NF-κB in HIV-1 vpr(+) or HIV-1 vpr(−)-virus-infected lymphocytes by indirect immunofluorescence.

(A) Infected PBL were fixed and cytospun on to slides, and stained for p65 subunit of NF-kB protein, as described in Materials and Methods. Infected Lymphocytes were detected by direct fluorescence of EGFP (Green), and the p65 subunit of NF-kB was detected using a polyclonal antibody (Red). Nuclei were identified by DAPI staining (Blue). Arrows in panel (overlay) indicate the presence of NF-kB within the infected cell. As indicated, the upper panels show the lymphocytes infected with HIV-1 vpr(+)/EGFP virus, and the lower panels show the cells infected with HIV-1 vpr(-)/EGFP virus. (B) Infected cells were identified by presence of EGFP florescence and bystander cells by the absence of EGFP florescence. Subcellular distribution of NF-kB was counted within the EGFP positive and EGFP negative cells in ten random fields (30 cells) for all multiple donors (N=4). The absolute cell numbers that are positive for NF-kB nuclear localization is presented. p value was derived using paired t test analysis by comparing HIV-1 vpr(+) and HIV-1 vpr(-) groups.

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3.4   DISCUSSION

Effective innate and adaptive immune responses are essential for control and elimination of viral infections. Many viruses, including HIV-1, have evolved ways to utilize viral antigens as a means of evading the host immune response [66, 86, 87]. There are increasing evidences for the role of HIV-1 accessory protein Vpr to modulate immune functions. Recently, we and others have shown that Vpr impaired the maturation of dendritic cells and subsequently dysregulated the activation and function of T cells [73, 74]. Despite the role of Vpr in inducing T cell apoptosis [88-90], it is unknown whether Vpr exerts immune dysfunction directly in infected T cells. Here, we have evaluated the direct effect of Vpr on T cell function independent of any interaction with APCs, using PBL and EGFP reporter containing X4 tropic (pNL43) HIV-1 vpr(+) and HIV-1 vpr(-) viruses.

The costimulatory molecules CD28 and CTLA-4 are the major players regulating the proximal TCR signaling events. Dysregulation of CD28 and CTLA-4 on both CD4+ and CD8+ lymphocytes has been well documented in HIV-1 infected patients and is related to progression of the disease [36, 37, 39, 67], and HIV-1 accessory proteins selectively regulate this process [67]. Our results indicate that Vpr differentially modulated CD28 and CTLA-4 molecules in infected T cells, in the presence of Nef and other viral proteins, indicating that HIV-1 proteins might function in an additive manner. The selective nature of Vpr-mediated downregulation is further supported by the observation that under similar conditions (same time points) Vpr did not affect the surface expression of CD25 or HLA-DR. This suggests that the differences seen in the expression level of CD28 and CTLA-4, in the presence of HIV-1 Vpr, are very specific and not due to global changes in the expression of cellular molecules or differences in the activation

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status of the infected lymphocytes. We next delineated the defect in downstream effector function (measured by IFN-γ production) in infected cells as an outcome of Vpr-mediated differential regulation of CD28 and CTLA-4. HIV proteins Env, Tat and Nef have been shown to directly activate some of the down stream factors, but most of these studies have been conducted using recombinant viral proteins, and the effects in the context of infection is limited [67]. Next we assessed the effect of Vpr on downstream effector molecule, IFN-γ in these cultures. HIV-1 Vpr not only reduced IFN-γ production in infected culture, it also further blocked the stimulation by potent stimulators, such as PMA and ionomycin that bypass the proximal signaling events and directly activate the downstream signaling factors [91, 92]. This clearly suggests that HIV-1 Vpr not only interferes with the proximal signaling in T cells, but also has additional inhibitory effects on the down stream effector molecules.

NF-kB is one of the major down stream factors involved in the regulation of secretion of IFÑγ upon TCR signaling [93]. HIV-1 Vpr has been reported to both activate and inhibit NF-kB mediated down stream effects, depending on the cellular context, Vpr forms, and activation status of the cells [31, 94]. In our current study, we have shown that Vpr inhibited the nuclear translocation of the p65 subunit of NF-kB. Activated NF-kB is required for antiviral effects. Collectively, the results presented in this study delineate the role of HIV-1 Vpr in the regulation of T lymphocyte function in the context of infection mainly by altering the level of proximal TCR signaling, as evident by downregulation of CD28 and simultaneous upregulation of CTLA4, and by blocking distal events (IFN-γ production) at the transcriptional level independently. These events initiated early during infection could not be reversed by restimulation as observed in PMA/ionomycin activation, suggesting that Vpr-mediated immune defects are not completely reversible. The observed Vpr-mediated inhibition of NF-kB

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translocation coupled with functional impairment of T cell IFN-g production could preferentially induce an immunosuppressive phenotype of infected T lymphocytes. Collectively, the results presented here delineate a role for HIV-1 Vpr in modulating the function of T lymphocytes during early stages of infection. Thus, HIV-1 Vpr might potentially utilize multiple mechanism(s) to evade the immune system, and the viral proteins could act in a coordinated manner to impair immune activation. Identifying the mechanism(s) involved in Vpr-mediated immune regulation will improve our understanding of viral function, and assist in the development of immunotherapeutics and antiviral strategies for HIV-1 infected individuals.

4.0 INTERACTION OF VPR DYSREGULATED T LYMPHOCYTES WITH NATURAL KILLER CELLS AND DENDRITIC CELLS: INDIRECT EFFECT OF VPR THROUGH INFECTED TARGETS ON IMMUNOSUPPRESSION AND VIRUS REPLICATION

4.1 INTRODUCTION

In the previous section, I have evaluated and discussed the direct effect of Vpr on infected and exposed/bystander CD4+ T cells. This section (aim#2), I propose to assess the effect of infected/exposed CD4+ T cells on other immune cells specially NK cells and DC. The rationale for this study is based on the established interaction of immune cells (T cell, DC and NK cells) either as part of the general surveillance mechanism or during the process of induction of effective response to invading pathogen, including HIV. These intercellular interactions are mediated by soluble and membrane bound factors as well as specific ligand-receptor molecules and is responsible for initiating innate and adaptive immunity. Although pathogens (viruses, bacteria) might target specific cell types, the host immune system has the capacity to effectively mount innate and adaptive response through these interactions and signaling mechanisms. HIV-1 is known to infect macrophages, DC and CD4+ T cells, however CD4+ T cells are the major targets for infection. Therefore, it is important to understand how infected T cells interact with NK cells or DC and how this cross talk alters the uninfected interacting partners and

subsequently the immune response and viral pathogenesis. The first part of this aim focuses on the consequence of T cell-NK interaction and the second part focuses on T cell-DC interaction as shown in Figure 9.
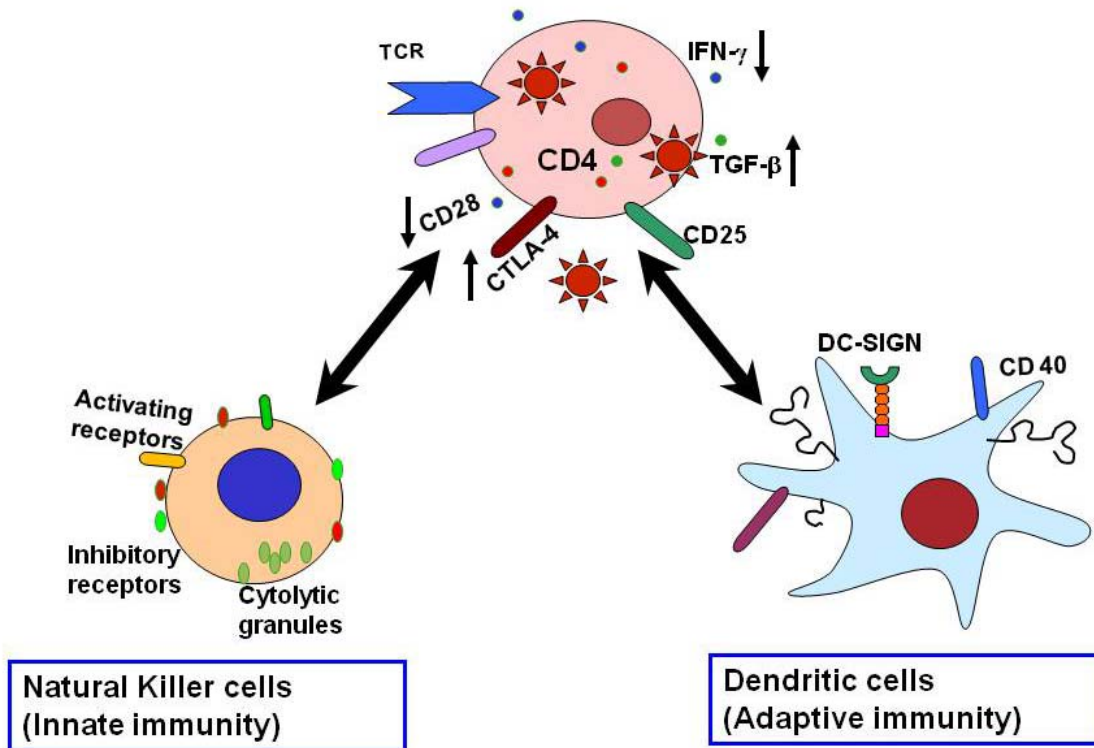
Figure 9. Schematic representation of T cell-NK cell and Tcell-Dendritic cell interaction.

4.2   PART I: T CELL-NK CELL INTERACTION AND ITS CONSEQUENCE IN VIRAL PATHOGENESIS

Natural killer (NK) cells are major effectors of the innate immune system. Although defective NK cell functions have been well documented in HIV-1 infected individuals [95-97], there is little information on the mechanisms underlying these defects. Understanding the basis of NK cell defects in HIV-1 infection could be important to development of effective immunotherapies

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and vaccines for HIV-1. Interestingly, it is not likely that these defects result from direct infection of NK cells, as NK cells are not considered to be major targets of HIV-1 infection and do not support virus replication, suggesting that NK cell dysfunction could be mediated through their interaction with infected and exposed target cells and/or by direct exposure to viral proteins present in the infected milieu. NK cells interact with immune cells (T cells, monocytes/macrophages and DC) through their cognate receptors / ligands and cytokine networks as a normal immunological process.

Dysregulation of NK cell function has a direct correlation with viral load [96, 98-102]. Early studies by our group and others demonstrated that despite the presence of normal NK cell numbers , the activities of NK cells are significantly compromised in HIV-1 infected individuals [103, 104]. There is also a distinct change in NK cell subset distribution during acute HIV-1 infection that preceded the progressive depletion of functionally active NK cells. HIV-1 infection is characterized by a dramatic increase in inhibitory receptors and loss of activating receptors, particularly NKp30 on NK cells, resulting in loss of NK cell activity.

NK cells interact with infected targets cells and with additional immune cell types during immune activation via ligand-receptor interactions. Pathogens, including HIV-1, are known to modulate the ligands present on the infect cells, thus avoiding reorganization by the innate immune system, as well as to suppress the adaptive immune response. Recent studies by several investigators have shown that HIV-1 infection alters expression of ligands in the infected culture, thus causing a defect in NK cell function through ligand-receptor interaction and signaling [105]. Furthermore, alteration in ligands for NK receptors (ULBP1, 2, 3, MIC A/B for NKG2D) also accounts for a defect in NK signaling cascades and cytolysis [106].

Although NK cells are regulated via direct and indirect mechanism(s), the role of virion associated proteins and the pathways involved in HIV-1-NK dysregulation have yet to be determined. To delineate the role of HIV-1 Vpr on NK cell function, through infected and exposed target cells, we assessed the ability of NK cells to kill target cells and to produce IFN-γ or express degranulation marker, CD107a, on challenge with K562 target cells. Additionally, we identified the T cell derived factors involved in this process. Results implicate the role of TGF-β and NK cell ligands in HIV-1 Vpr mediated NK cell dysregulation indirectly through the infected / exposed T cells. This affect is predominant during early phases of infection or exposure. Thus, a more indepth understanding of Vpr and its role in HIV-1-immunopathogenesis should lead to significant narrowing in our gaps in knowledge regarding HIV-1 pathogenesis and immunosuppression.

4.3   MATERIALS AND METHODS

4.3.1   Cell Culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from normal donors using Ficoll-Hypaque gradient centrifugation. PBMC were cultured in RPMI containing 1mg/ml PHA-P and 200U IL-2 .In certain experiments, CD4+ lymphocytes were isolated by negative selection from PBMC, more than 95% of isolated cells were CD4+ as tested by flow cytometry. For NK studies, pure NK was isolated by positive selection using anti-CD56 antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as described previously [74]. In some experiments, NK cells were isolated by negative selection.

4.3.2 Plasmids, virus preparation and infection

HIV-1wt and HIV-1ΔVpr plasmids were obtained from NIH, HIV AIDS Research Reagent Program and Dr. David N.Levy, University of Alabama. HEK293T cells (2×10^6 per plate) were transfected with 10μg of HIV-1 constructs by calcium phosphate precipitation method [74]. Forty-eight hours post transfection supernatants were collected, filtered through a 0.45-μm filter to remove cellular debris, and centrifuged at 22,000 rpm for 1 h. Virus pellets were resuspended in PBS and stored in aliquots at -80°C for subsequent assays. Virus titers were measured by p24 enzyme-linked immunosorbent assay (ELISA), and multiplicity of infection (MOI) was assessed by standard TZM-bl assay. On the day of isolation the cells were exposed to either HIV-1wt or HIV-1ΔVpr virus, the viral dose equivalent to 100ng of p24 per 10 million cells was used.

4.3.3 NK cell mediated target cell lysis assay.

PBMC (1×10^7 cells in a final volume of 1ml) were infected with HIV-1wt and HIV-1ΔVpr virus, or exposed to Vpr , other HIV-1 viral proteins or control protein, as described above, were used to assess NK-mediated cytolytic function using K562 cells as targets (kindly provided by Dr. Pawel Kalinski, University of Pittsburgh). Briefly, infected/exposed PBMC (12, 24, 48, 72, 96 hrs and 6 days post infection) were cocultured with K562 at an effector : target ratio of 1:1 and 10:1 in triplicate. In certain experiments, Cytotoxicity was assessed using a non-radioactive colorimetric assay measuring lactate dehydrogenase (LDH) released from lysed target cells following the manufacturer's instructions (Cytotox96 non-radioactive cytotoxicity assay, Promega, WI). Following four hrs coculture at 37°C in a 96-well flat bottom microtiter plate,

50µl aliquots of cell-free supernatants were transferred in a new assay plate and incubated at room temperature with 50µl reconstituted LDH substrate mix in the dark for 30 minutes. Target cells and effector cells incubated separately for the same period were used as target and effector spontaneous controls, respectively. Target cells treated with 0.8% Triton-X lysis solution for 45 minutes prior to harvesting the supernatants were served as the target maximum. Finally, the assay was terminated by adding 50µl stop solution and the release of LDH was measured at 490nm using an ELISA plate reader. Percent cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental-Effector Spontaneous-Target Spontaneous}}{\text{Target Maximum-Target Spontaneous}} \times 100$$

In some experiments, flow cytometry based NK cell lysis assay was used.

4.3.4   NK cell degranulation assay.

To quantitate the cell surface expression of CD107a, infected or Vpr exposed PBMC and PBL (total 1×10^6/mL) were washed twice in PBS, and activated with K562 target cells (PBMC: K562=1:1) in a total volume of 1ml prewarmed culture media following centrifugation for 5 minutes at 1200 rpm to facilitate the contact of cells. PBMC stimulated for one hour with K562 were further incubated with 6µl monensin (BD Bioscience, Mountainview, CA) at a final concentration of 6µg/ml for additional three hrs prior to the surface staining for CD107a. NK cells were stained with anti CD3-ECD, anti CD56-PC5 (Beckman Coulter, Miami, Fl) and anti CD107a-FITC (BD Bioscience) antibodies for one hour in FACS buffer (2% FBS in PBS)

containing 5mM EDTA on ice and analyzed for surface expression of CD107a by flow cytometry as described [107].

4.3.5   Detection of intracellular IFN-γ.

Uninfected and infected PBMC and PBL (1×10^6 cells/mL) were further stimulated with K562 at an effector: target ratio of 10:1 for 6 hrs in the presence of 6μl of monensin (BD Bioscience). Production of IFN-γ was detected in fixed and permeabilized cells (cytoFix-cytoPerm kit, BD-Biosciences, CA) by intracellular staining using PE-conjugated primary antibodies or isotype matched control (Caltag, Burlingame, CA) following surface staining with CD56-PC5 and CD3-ECD antibodies to distinguish CD3-/CD56+ NK cells within the total PBMC. Briefly, cells were washed three times with FACS buffer and surface markers were stained with anti CD56-PC5 and anti CD3-ECD for one hour. Intracellular IFN-γ staining was performed at room temperature for 60 minutes using 5μl PE-conjugated anti-IFN-γ antibodies (Coulter, FL), followed by two washes in Perm-Wash buffer. The cells were gated in ECD-, PC5+ channels to quantitate the expression of intracellular cytokine in specific NK cell subpopulations (CD3-/CD56+ cells) and analyzed by flow cytometry as described [102, 108].

4.3.6 TGF-β ELISA

Culture supernatants were collected at different time points post infection or post treatment were measured by ELISA for TGF-β using BD Opti-EIA kits as per the manufacturer's instructions (BD Biosciences).

4.3.7 Flow cytometry

To confirm the purity of cells, we examined the phenotype of the cells by flow cytometry following staining with specific antibody. Surface expression of NK ligands, MHC class I molecules were evaluated by flow cytometry. Intracellular staining was done to evaluate the production of IFN-γ. Intracellular cytokine staining was carried out following the standard staining protocol. Cells (1×10^6) at the mentioned time point were stimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) in the presence of GolgiStop (2 µl/ml) for a duration of 8 h, followed by incubation at 4 °C for 6 h. Intracellular staining was done with IFN-γ or IgG1 isotype control using the same protocol as described above. Samples were analyzed using Epics-XL (Beckman Coulter, Miami, FL) or FACS Canto, with minimum of 20,000 gated events acquired for each sample, and the mean fluorescence intensity (MFI) was calculated using Flow Jo software.

4.3.8   Statistical analysis

Results were analyzed using the student t test. A p value less than 0.05 was considered as statistically significant.

4.4   RESULTS

4.4.1   Effect of HIV-1 vpr on NK cell function during HIV-1 infection.

HIV-1 Vpr is known to dysregulate the cells of the immune system, but the ability of vpr to affect NK cell function is not clearly defined. Hence we evaluated the effects of HIV-1 vpr on NK cells in PBMC cultures infected with either HIV-1 wild type (HIV-1 wt) virus or HIV-1 vpr defective (HIV-1Δvpr) virus or PBMC exposed to recombinant Vpr or control protein. The effectiveness of NK cells present in the infected or protein exposed cultures to lyse target cells and the ability of NK cells to express degranulation marker, CD107a, or their ability to secrete IFN-γ in response to challenge with target cells was evaluated. Infected and protein treated PBMC were cocultured with K562 targets at ratios of 1:1 and 10:1; target cell killing was measured by flow cytometry based NK cell lysis assay as previously described. NK cells in PBMC infected with HIV-1 wt virus induced about 3% target cell lysis at a 1:1 ratio, whereas NK cells in PBMC infected with HIV-1Δvpr virus mediated 6% target lysis at the same ratio (Fig.10). A similar difference in specific lysis of targets was displayed by NK cells from
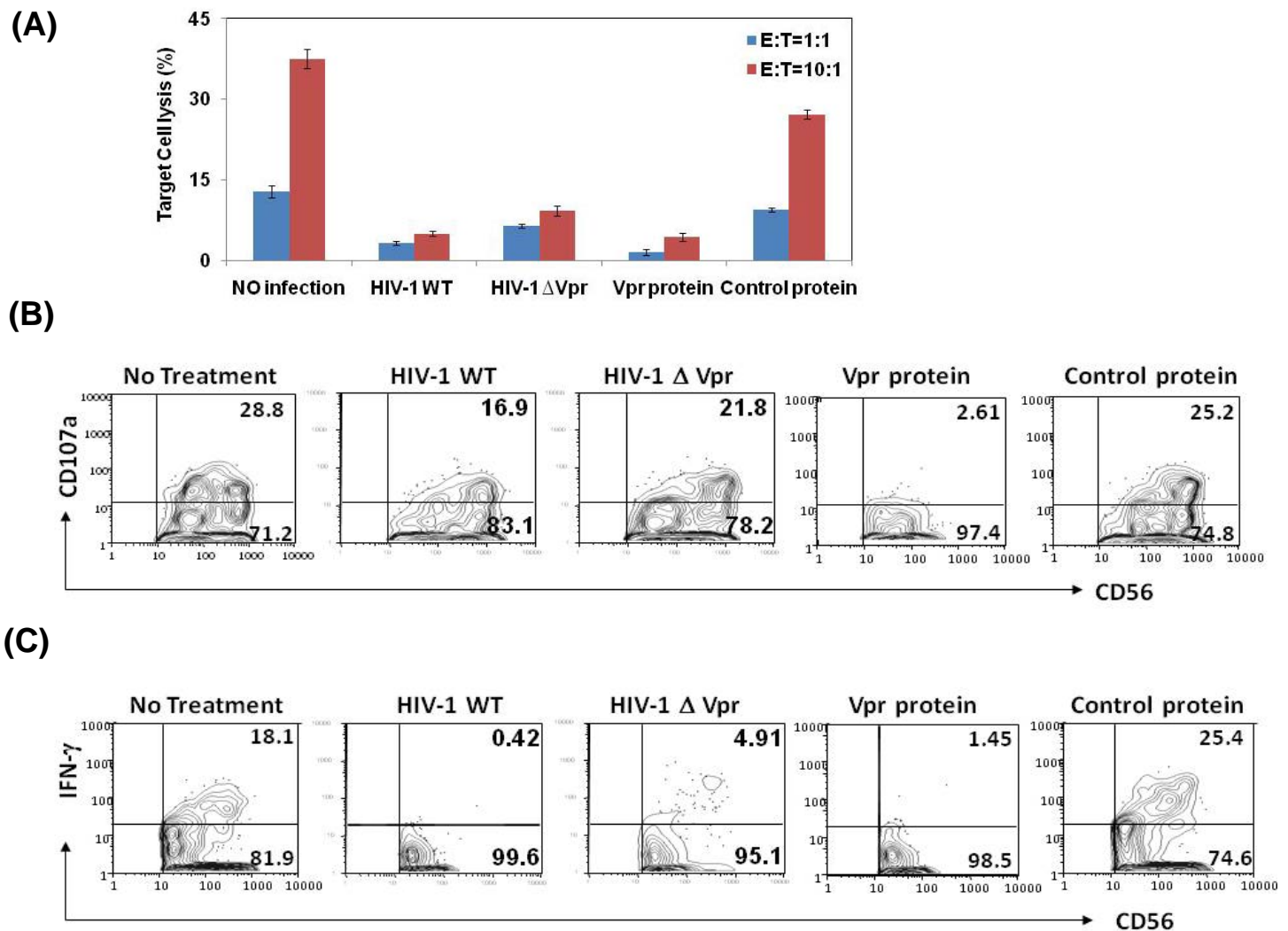
Figure 10. HIV-1 Vpr inhibits NK cell function in infected and Vpr protein treated PBMC cultures.

PBMC was infected with either HIV-1 wt or HIV-1Δvpr virus or exposed to Vpr or control protein, four days post infection/ exposure, the NK cells were evaluated for (A) specific target cell lysis, (E:T=1:1 and E:T=1:10) (B) CD107a expression (C) IFN-γ production in response to K562 cell line (NK:K562 1:5).

HIV-1 wt virus-infected PBMC or HIV-1 Δ vpr virus-infected PBMC at 10:1 effector/target ratios. Vpr protein was also able to inhibit the NK cell lysis at both the ratios.

Evaluation of NK cells from these cultures to express degranulation marker, CD107a, or secrete IFN-γ production further confirmed the effect of Vpr to inhibit NK cell function. As shown in Fig.10B and Fig.10C, there was a substantial reduction in CD107a expression (1.5-10

fold) and intracellular IFN-γ production (4-20-fold) in CD3⁻ CD56⁺ gated NK cells. A dose dependent inhibition of NK cell functions was seen with the treatment of recombinant Vpr protein (data not shown). Similar results were observed in multiple donors (N=4). Together, these results suggest that HIV-1 Vpr inhibits NK cell function both in HIV-1 infected and Vpr protein treated cultures.

4.4.2   Effect of vpr dysregulated CD4+ T cells on NK cell function during HIV-1 infection

Above results indicate that HIV-1 Vpr adversely affects NK cell function in PBMC cultures in vitro , we wanted to evaluate the role of CD4+ T cells derived factors in Vpr mediated NK cell dysfunction. Pure CD4+ T cells were infected with wild type or Vpr defective HIV-1 virus or exposed to recombinant Vpr or control protein. Three days post infection/treatment , the CD4+ T cells were cocultured with autologous NK cells at a ratio of (T cell : NK cell = 5:1) for twenty four hours. As a control, HIV-1 virus or the proteins were added to pure NK cell cultures. Following 24 hours of coculture, NK cells where challenged with K562 target cells and the surface expression of CD107a and IFN-γ production in CD56+CD3- NK cells was evaluated by flow cytometry. Results indicate that there was a significant reduction in CD107a expression (2-3 fold) and intracellular IFN-γ   production (1.5-20 fold) in CD3⁻ CD56⁺ gated NK cells. At the same time point, no significant effect of Vpr was observe in CD107a expression and IFN-γ production in pure NK cultures containing HIV-1 virus or Vpr protein, suggesting that the effects seen in the CD4+ T cell: NK cocultures is mainly due to effect of CD4+T cells altered by Vpr.
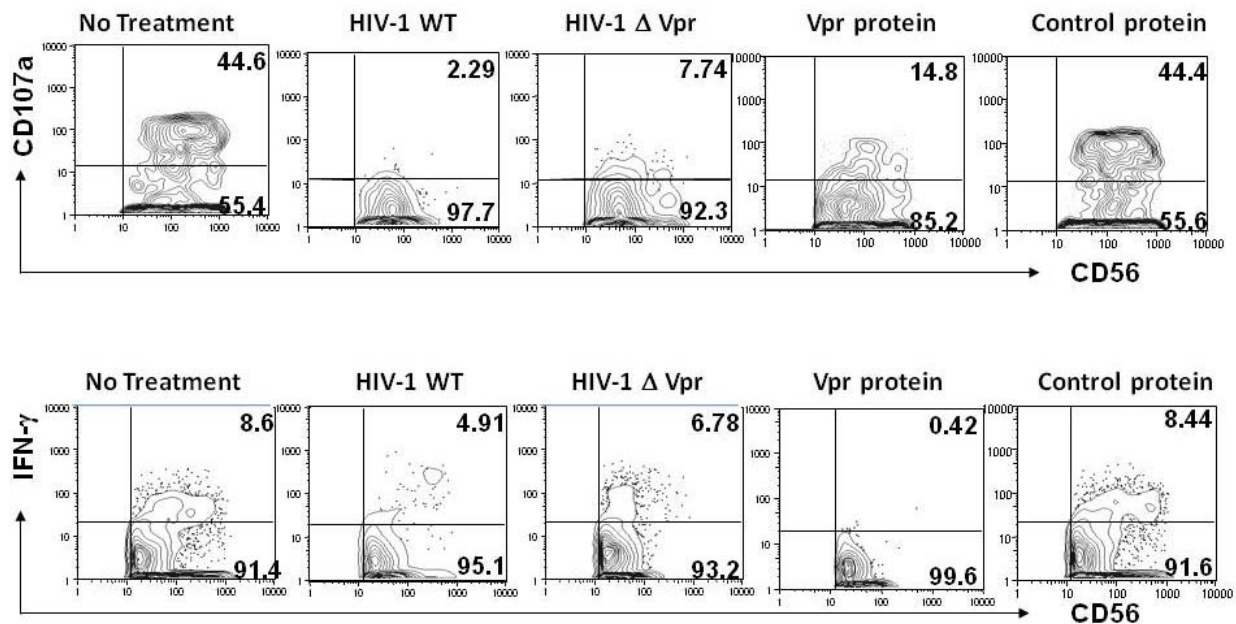
Figure 11. HIV-1 Vpr inhibits NK cell function in infected and Vpr protein treated CD4+ T cell-NK cell cocultures.

*CD4+T cells isolated from PBMC by negative selection, was exposed to either vpr or control protein for 60 hours. At this time point, pure autologous NK were cocultured with the CD4+ T cells for 24 hours. After 24 hours of coculture, the NK cells were evaluated for (**A**) CD107a expression and (**B**) IFN-γ production in response to K562 cell line (NK:K562 1:5).*

4.4.3 Role of soluble factors in Vpr mediated inhibition of NK cell activity

The ability of HIV-1 Vpr to inhibit NK cell function in NK –CD4+ T cell cocultures but not in pure NK cell cultures indicate a role for CD4+ T cell derived soluble and membrane bound factors. TGF-β is one the T cell derived cytokines that is known to affect NK cell survival/proliferation and functions. Results from previous chapter, indicate that Vpr increases the surface expression of CTLA-4 on infected cells. CTLA-4 is strongly associated with increased production of TGF-β. Hence we evaluated the TGF- β level in supernatants collected from PBMC infected with HIV-1 wt or HIV-1Δvpr virus at 96 hours post infection. Results from figure 12, indicates that Vpr significantly increased the production of TGF-β in multiple donors.

Next, to confirm the role of TGF- β in the Vpr-induced NK dysfunction, we set up experiment to neutralizing the overproduction of TGF-β with anti-TGF-β antibody. PBMC infected as described above were treated with anti-TGF-β (1 µg/ml; R&D Systems) or Isotype control at 12 h post infection and maintained for an additional 3 days. As a positive control, cells were also treated with recombinant TGF-β (5 ng/ml; R&D Systems). NK cell-mediated cytotoxicity was measured in all the groups. Results indicate that treatment of HIV-1 wt virus-infected PBMC with anti-TGF-β increased NK cell-mediated lysis to 60%, compared to 35% lysis observed in Isotype treated control. Conversely, single exposure to recombinant TGF-β also inhibited NK cell-mediated killing of target cells in uninfected and HIV-1Δvpr virus-infected PBMC under similar conditions. However, in the case of HIV-1wt virus-infected culture, no additional inhibition was observed. Similar results were observed in Vpr protein-treated PBMC (data not shown). Together, these results suggest that in addition to TGF-β, there may be other factors that might also play a role in NK cell dysfunction.
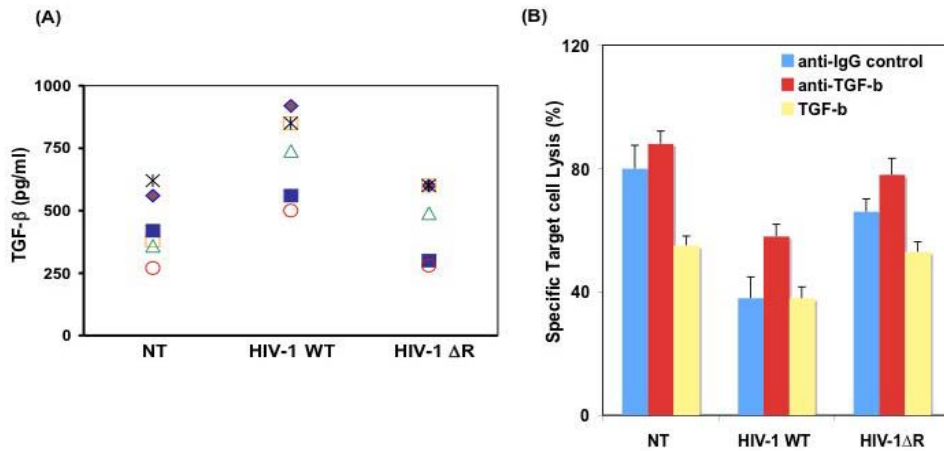
Figure 12. Role of TGF-β in Vpr mediated NK cell dysfunction.

PBMC infected with HIV-1wt or HIV-1Δvpr virus were cultured as described in Materials and Methods. Supernatants were collected and assessed for (A) TGF- β production by ELISA. Dot blots represent results from multiple donors (n = 6). (B) PBMC were pretreated with anti-TGF- β 2 h prior to infection or treated with TGF- β 12 h post infection. NK cell function was assessed by measuring the percent specific lysis in different groups at an effector/target cell ratio of 10:1. Data are the representative of one of three independent experiments.

4.4.4 Role of membrane associated factors from infected/exposed target cells in Vpr mediated NK cell dysfunction.

Inability to reverse NK cell function by anti-TGF-β antibodies suggest that additional factors from T cells may also involve in NK cell dysfunction. Therefore we next evaluated the role of membrane bound factors that are known to play a role in T cell-NK cross talk. Previous studies have established that NK cells interact with T cells through the ligands present in T cells. To assess the effect of Vpr on expression of the NK cell ligands in infected/exposed T cells, PBMC infected/exposed to HIV-1wt, HIV-1ΔVpr, Vpr protein, other HIV-1 proteins or control protein as described in methods, was evaluated for surface expression of NK ligands, MIC A/B, hULBP-1 and hULBP-2 by flow cytometry.

Results from multiple donors show a significant increase in the expression of MIC A/B, hULBP-1 and hULBP-2 on the virus exposed lymphocytes during HIV-1 infection with the wild type virus, and there was a lesser degree of increase on day 2 post infection in case of the virus which lacks HIV-1 Vpr. There was no difference in the expression of MHC class 1 in the virus exposed cells. Results were more significant when the lymphocytes were exposed to pseudo virus particles containing Gag, vsv-G envelope and HIV-1 vpr. The role of HIV-1 Vpr in the induction of MIC A /B is more significant in the early stages of infection, and the early response can be attributed to the release of Vpr incorporated in the virus particle.

(A)
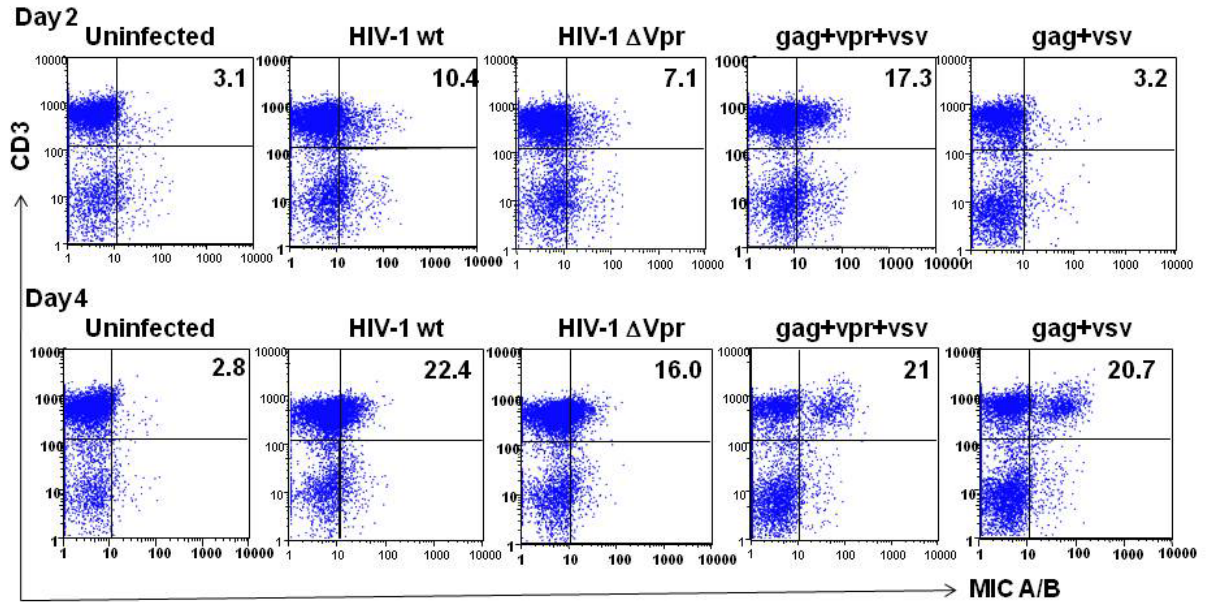
(B)
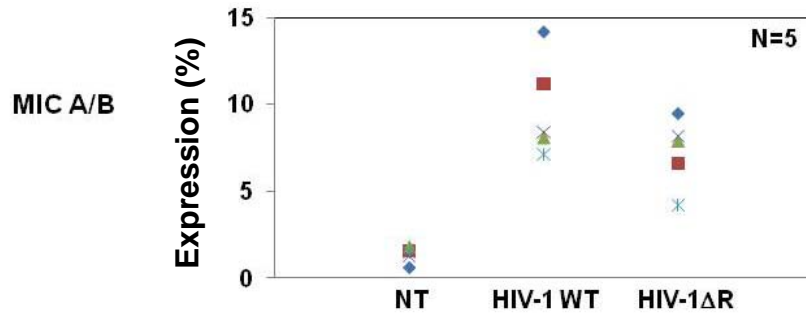
(C)
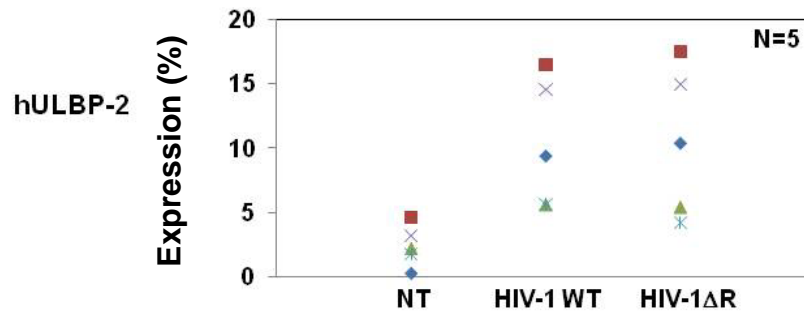
Figure 13. Effect of HIV-1 Vpr on surface expression of NK cell ligands in T cells.

*(**A**)PBMC obtained from healthy donors where cultured in RPMI containing 1mg/ml PHA-P and 200U IL-2 , on the day of isolation the cells were exposed to either HIV-1 wt - wild type HIV-1 virus; HIV-1DVpr - HIV-1 virus in which Vpr is deleted; gag+Vpr+vsv – pseudovirus particle having HIV-1 gag, HIV-1 vpr and vsv-G envelope; gag+vsv – pseudovirus particle having HIV-1 gag and vsv-G envelope, viral dose equivalent to 200ng of p24 per ten million cells was used. Following two days (upper panel) or four days (lower panel) of exposure the cells were stained with CD3 and MIC A/B specific monoclonal antibodies and analyzed by flow cytometry. Dot blots represent results from multiple donors (n = 5) for (**B**) MIC A/B (**C**) hULBP-2*

To specifically delineate the role of Vpr, we used recombinant Vpr protein and evaluated the role of Vpr protein to modulate the surface expression of NK activating receptor ligands. CD4+ T cells were isolated by negative selection from PBMC obtained from voluntary donors and exposed to vpr, HIV-1 viral proteins or control protein (100nM or 10nM). Additionally AT-2 treated virus was used to confirm the effects were due to virus exposure. At regular time points the surface expression of NK ligands MICA/B, huLBP-1, huLBP-2 was evaluated by flow cytometry. Figure 14, shows the surface expression of the NK activating receptor ligands and MHC class I (A,B,C), at 60 hours following exposure to the vpr or control protein from one of the representative donor. There is an upregulation in the surface expression of NK ligands MICA/B, hULBP-2, hULBP-3 in the vpr protein exposed culture, where as the control protein did not have any effect. Further the vpr protein specifically induced the surface expression of the NK activating ligands MICA/B, hULBP-2, hULBP-3, without any effect on the NK inhibitory ligands MHC class I A,B,C. Similar results were seen in multiple donors (N=3).

(A)

(Continued below)

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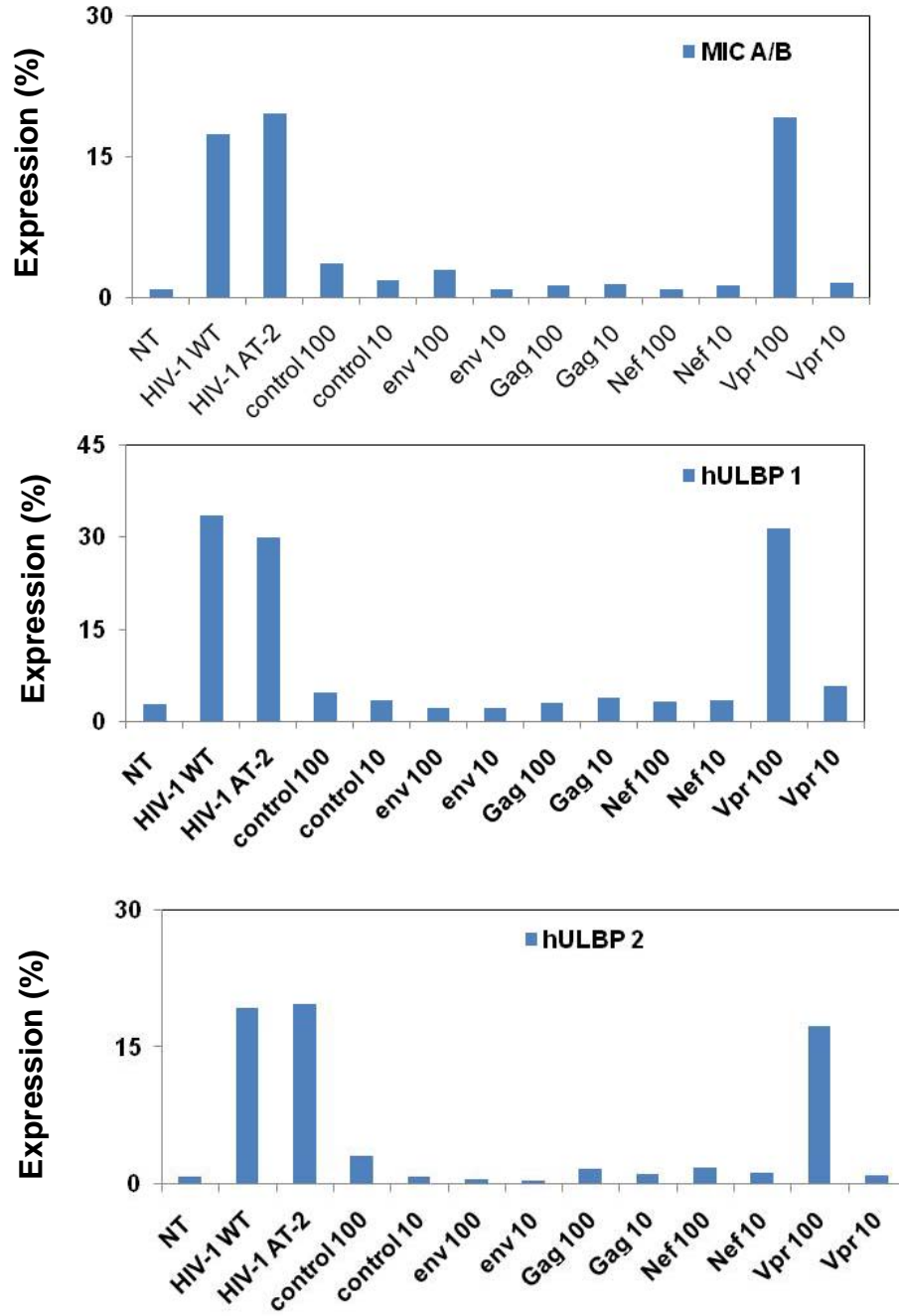
(B)
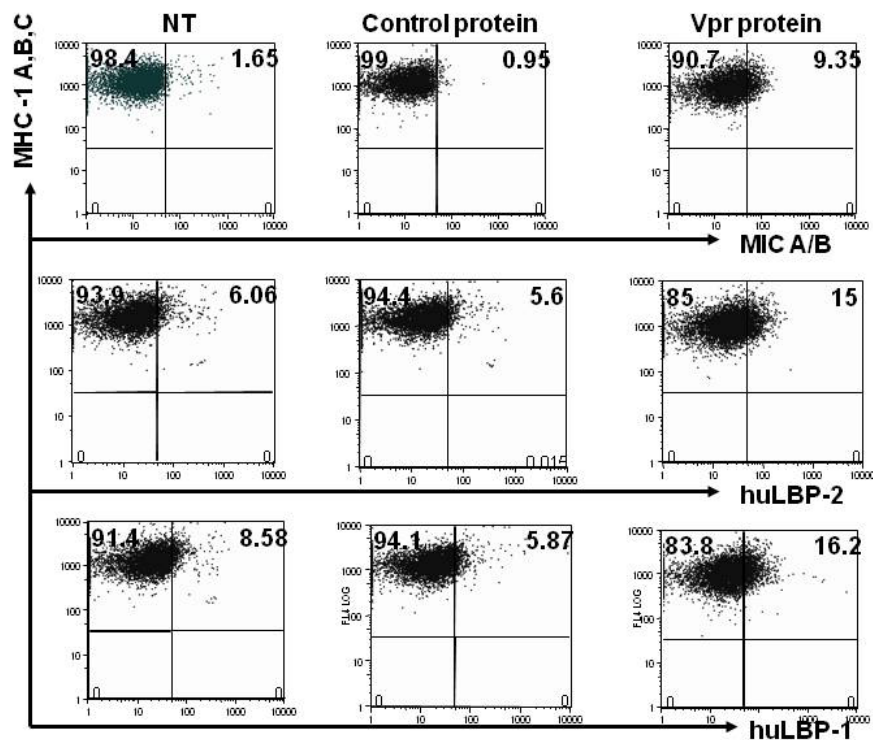
Figure 14. Upregulation of NK activating receptor ligands by HIV-1 Vpr protein.

CD4+ T cells were isolated by negative selection from PBMC from voluntary donor, and exposed to 5nM of Vpr or control protein. Sixty hours following exposure to the vpr or control protein, the cells were stained for surface expression of NK ligands, MIC A/B, hULBP-2 or hULBP-3 and MHC class I (A,B,C) and analyzed by flow cytometry.

4.4.5 Upregulation of NK receptor ligands on T cells is associated with downregulation of NK activating receptor, NKG2D.

Results from previous experiments show that Vpr has a role in upregulation activating receptor ligands, without affecting the expression level of NK inhibitory receptor ligands, MHC class I A,B,C molecules. Results from tumor biology indicate that cancer cells employ upregulation of NK activating receptor ligands as a mechanism to evade NK cell lysis. Chronic interaction of NK activating receptor with their cognate ligands induces tolerance to effective signal transduction and impairs normal calcium signaling and dysregulates NK lysis and IFN-g production, there is a strong association of this phenomenon with NK activating receptor down regulation. The ligands

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evaluated in this study, MIC A/B, hULBP-1 and hULBP-2, bind to the activating receptor, NKG2D present on the CD56+CD3-NK cells. Hence we evaluated the expression level of NKG2D on the NK cells present in the infected / exposed cultures in the context of role for HIV-1 Vpr. Results show that up to 90% of normal cells express NKG2D on their surface, during HIV-1 infection there is a decrease in the surface expression of NKG2D and the percentage of NK cells expressing NKG2D. HIV-1 Vpr seems to have a role and the Vpr protein is able to reduce the surface expression to 58%. Similar results were observed in multiple donors (N=3).
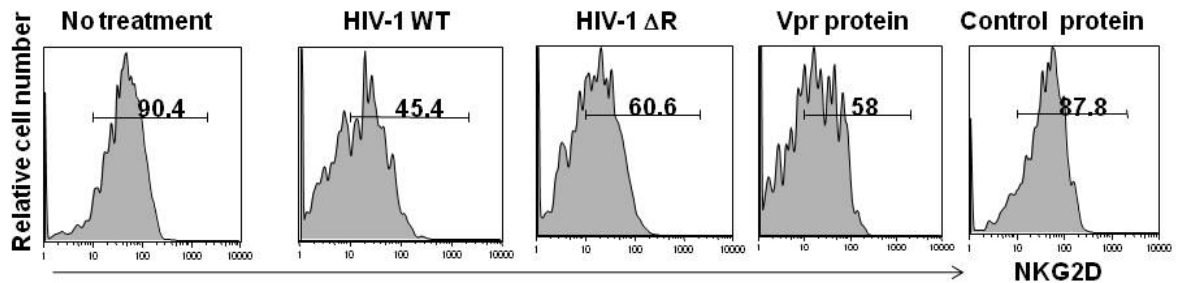
Figure 15. Effect of HIV-1 Vpr on NK activating receptor, NKG2D.

CD4+T cells isolated from PBMC by negative selection, was infected with HIV-1wt or HIV-1 Δvpr virus; or exposed to either vpr or control protein for 60 hours. At this time point, pure autologous NK were cocultured with the CD4+ T cells for 24 hours. After 24 hours of coculture, the NK cells were evaluated for NKG2D expression by flow cytometry.

4.5   DISCUSSION

NK cells mainly belong to innate immune system but have a central role in the induction of both innate and adaptive immune responses. HIV-1 infection is associated with loss of NK cell function both in vivo and in vitro. There is a direct correlation between viral load during HIV-1 infection and loss of NK cytotoxicity function, suggesting a role for viral antigens in the NK cell dysfunction. Here, we present results indicating that HIV-1 Vpr modulates NK cell functions and mechanistically understand the basis of Vpr mediated impairment of NK cell function. Results

from pure CD4+ T cells cocultured with autologous NK cells indicate a role for T cell factors in Vpr mediated NK cell dysfunction. We observed an increase in production of TGF-β and also a increase in surface expression of NK cell activating receptor ligands on T cells. This was associated with down regulation of activating receptor, NKG2D on NK cells present in the cultures. Similar phenomenon has been observed in Tumor cells and there is a direct correlation between the progression of the tumor, the level of NK activating receptor ligand expression and loss of NK cell function. HIV-1 virus seems to have duplicated a similar mechanism to evade the innate immune response mediated by NK cells.

HIV-1 Vpr is incorporated into Virus particles in significant amount and is delivered to the immune cells, early during infection. Our results support that HIV-1 virus associated Vpr exposure is sufficient to upregulate the NK activating receptor ligands and dysregulate NK cell function. The above results indicates that HIV-1 Vpr contributes to the loss of innate immune response seen early during HIV-1 infection. Understanding the molecular mechanisms involved in this process will aid in development of novel therapeutics, which can enhance the innate and adaptive immune response during HIV-1 infection.

4.6   PART II - T CELL –DC CROSS TALK AND ITS CONSEQUENCE IN VIRAL PATHOGENESIS

4.7   INTRODUCTION

Dendritic cells (DC) are one of the major professional antigen presenting cells (APC). DC have the ability to present both extracellular and intracellular antigen, to T cell through MHC molecules. Immature DC are specialized in antigen uptake and processing, the signals provided by the interacting antigen specific T cell and / or the signals emerging from the innate immune system, transform the immature DC to mature DC. Mature DC are highly specialized in antigen presentation and express co-stimulatory molecules, CD80, CD83, CD86, CD40 and others, which provide optimal signals required for T cell proliferation and activation. Mature DC also produce an array of cytokines, which provide the optimal environment, required for T cell differentiation and survival. T cells also express their own set of costimulatory molecules, CD28, CTLA-4, CD40L, and others, which interact with their counterparts on DC, and trigger bi-directional signaling in both T cell and interacting DC. These signals are critical in determining the outcome of immune response to the presenting antigen. Interaction of CD28 with the costimulatory molecules CD80 and CD86 on DC induces the immuno-stimulatory signals in DC where as CTLA-4 induces tolerogenic signals in DC mediated by IFN-g driven expression of immunosuppressive tryptophan catabolism[40, 41]. HIV-1 accessory proteins, including Vpr are known to directly dysregulate DC maturation and alter the cytokine profile [3]. Additionally, HIV-1 enhances viral transmission by converting the immunological synapse to a virological synapse between the interacting antigen presenting cell (APC) and T cell. Studies also report that Vpr is required for efficient nuclear translocation of Pre-Integration Complex (PIC) in non-dividing cells. Results from chapter 1, indicate that HIV-1 Vpr differentially

modulates the expression of costimulatory molecules, CD28 and CTLA-4 on infected T cells and alters the cytokine profile, hence we evaluated the role of HIV-1Vpr on the outcome of T cell – DC interaction. To address this, we cocultured HIV-1 WT and HIV Δ Vpr infected T cells with naïve DC, either directly or separated by a transwell, and evaluated the outcome of the interaction. The separation of Tcell and DC by transwell of 0.6μm will help us to determine the role of secretary factors on the outcome of interaction, independent of membrane bound factors.

Results presented here indicate that even though HIV-1 Vpr did not have a significant effect to alter expression of costimulatory molecules, CD80, CD83 or CD86 on cocultured naïve DC, but we observed that cell-associated virus was taken by DC and infected DC as early as 12 hours and was maintained for more than six days. Infection of DC via infected T cell is dependent on T cell-DC contact and is independent of viral envelope and DC-SIGN or other known HIV-1 receptors. Furthermore, the percentage of DC infection is directly correlated with the ability of DC to acquire cell-associated antigen, suggesting DC could acquire virus from the infected T cells through an antigen uptake process. Collectively, these studies for the first time indicate that HIV-1 taken up by the DC through the antigen uptake mechanisms establishes *cis* infection in DC.

4.8   MATERIALS AND METHODS

4.8.1   Cell Culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from normal donors using Ficoll-Hypaque gradient centrifugation. CD14+ monocytes were purified by positive selection using anti-CD14 monoclonal antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as described previously [74]. The purity of CD14+ cells was tested by flow cytometry following staining with CD14 antibody, and the results indicate >98% of isolated cells were CD14 positive (data not shown). To obtain monocyte-derived DC (MDDC), CD14+ cells (0.5x106 cells/ml) were cultured in 60-mm culture plates in a total volume of 10 ml of medium containing 25ng/ml IL-4 (R&D Systems, Minneapolis, MN) and 50ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems). Half the volume of medium was replaced every other day throughout the entire culture period. MDDC (7 day old) were stimulated with LPS (Sigma-Aldrich) 1μg/ml, and maturation of MDDC was confirmed by phenotypic and functional analysis. The flow through during the CD14+ selection of PBMC (same donor) was collected, and the purity of the lymphocytes (PBL) was tested by flow cytometry using CD14 and CD3 antibody. More than 95% of isolated cells were CD14− (data not shown). PBL (1x107/ml) were stimulated with anti-CD3 (OKT3) antibody (10μg/ml)-coated flasks along with soluble anti-CD28 antibody (BD Pharmingen clone 28.2) (1μg/ml) for 3 days as described [75] and cultured in media containing rIL-2 (5 U/ml). CD4+ lymphocytes were isolated by negative selection from PBMC, more than 95% of isolated cells were CD4+ as tested by flow cytometry. Stimulation and infection of CD4+ T cells was performed in a similar way as PBL. Jurkat T cell line JJK, HeLa-T4 and HEK 293T cells were maintained in

appropriate growth media. For NK studies , pure NK was isolated by positive selection using anti-CD56 antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) as described previously [74]. In some experiments, NK cells were isolated by negative selection.

4.8.2   Plasmids, virus preparation and infection

The construction and characterization of HIV-1 wt-EGFP proviral plasmid has been previously described [109]. Briefly, Enhanced Green Fluorescence Protein (EGFP) gene was inserted in the nef open reading frame of pNL4.3 proviral plasmid and the expression of nef was driven by ECMV Internal Ribosomal Entry Site (IRES). For studies involving envelope deficient HIV-1 E-EGFP virus, NdeI site in the envelope of HIV-1wt-EGFP proviral plasmid was mutated by filling and re-ligating the blunt ends. This introduced multiple stop codons in the reading frame of env after the end of vpu reading frame, and deleted the expression of env. HEK293T cells (2×10^{6} per plate) were transfected with 10μg of HIV-1wt-EGFP construct by calcium phosphate precipitation method [74]. Forty-eight hours post transfection supernatants were collected, filtered through a 0.45-μm filter to remove cellular debris, and centrifuged at 22,000 rpm for 1 h. Virus pellets were resuspended in PBS and stored in aliquots at -80°C for subsequent assays. Virus titers were measured by p24 enzyme-linked immunosorbent assay (ELISA), and multiplicity of infection (MOI) was calculated by infecting Jurkat cells for 24 hours and assessed by flow cytometry or by standard TZM-bl assay. PBL and Jurkat were infected with the HIV-1wt-EGFP reporter virus at a MOI of 0.5. Twelve hours post infection, virus was removed by washing, and cells were maintained in appropriate media. For infection of HEK293T and HeLa-T4 cells, the HIV-1wt-EGFP reporter virus was pseudotyped with VSV-G envelope and the cells were infected at a MOI of 1.0. Six hours post infection, the virus was removed by washing and

the cells were maintained in growth media. VSV-G pseudotyped HIV-1ΔE-EGFP virus was produced by co-transfecting HEK293T cells with VSV-G and HIV-1ΔE-EGFP constructs by calcium phosphate precipitation method [74]. Forty-eight hours post transfection supernatants were collected, processed and the virus was quantitated as described above. PBL and Jurkat were infected with the HIV-1wt-EGFP reporter virus at a MOI of 0.5. Twelve hours post infection, virus was removed by washing, and cells were maintained in appropriate media. Transfection of HIV-1ΔE-EGFP construct in the absence of VSV-G produced non-infectious virus like particles. Similarly the virus particles released from the T cells in the supernatant were not infectious as evaluated by standard TZM-bl assay.

4.8.3 Transfection of Jurkat

Jurkat T cell line JJK (CD4+/CD28+), were nucleofected with pEGFP, plasmid expressing EGFP using Amaxa nucleofector system, Amaxa Biosystems, Gaithersburg, MD following manufacturer's instructions. Briefly, the cells were washed and resuspended in RPMI medium without any supplements at a concentration of 5×10^6, and 5μg of plasmid was used to transfect the cells using appropriate settings. Following nucleofection, cells were maintained in RPMI supplemented with 10% FBS and 1% L-glutamate with no antibiotics.

4.8.4 Flow cytometry

In coculture experiments, doublet differentiation was applied to gate on single cells. Surface staining was performed for DC-SIGN, in some of the experiments surface staining of CD3, or CD28 was also included. Briefly, at indicated time points cells were washed twice with cold PBS

(pH 7.2) containing 5% FBS and incubated with respective fluorochrome conjugated antibody or isotype control for 1 h at 4 °C. To minimize cell aggregates, 5mM EDTA was included in the FACS buffer. Samples were fixed with 2% formaldehyde for 1 hr and analyzed using Epics-XL (Beckman Coulter, Miami, FL) with minimum of 20,000 gated events acquired for each sample. Flow Jo software was used to analyze the results.

4.8.5   Fluorescence labeling

Cells were labeled with membrane labeling dye PKH26 (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, the cells were washed twice in PBS and resuspended in Diluent C at a concentration of $2x10^6$ cells/ml. Cells were added to an equal volume of PKH26 (4µM) in Diluent C, and incubated for 5 min at room temperature. An equal volume of FCS was added following which the cells were resuspended in complete medium and washed four times. The stained cells were incubated in appropriate medium for 4 hours before using them in coculture experiments.

4.8.6   Inhibition and blocking assays

Two hours prior to coculture with infected cells, immature or mature MDDC were pretreated with the inhibitor/blocker. Anti-DC-SIGN, and anti-DC-SIGN-R antibody were obtained from R&D Systems; Integrase inhibitor (118-D-24), T-20 Fusion inhibitor, TAK 779, AMD3100 were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; and all other inhibitors were obtained from Sigma-Aldrich.

4.8.7 Immunofluorescence

At indicated time points, cells were adhered to glass slides, and fixed using 3.7% formaldehyde at room temperature for 10 min, and washed with PBS. Following three washes, slides were blocked with PBS containing 5% FBS. Surface staining was performed with primary antibody (DC-SIGN or CD28) (1:100 dilution, R&D Systems, or BD Biosciences) for 1 hr at 4 °C. Cells were washed 3 times with wash buffer, and incubated with rabbit anti-mouse IgG Rhodamine (RRX) (1:400; Jackson ImmunoResearch, West Grove, PA) or donkey anti mouse Cy3 (1:1000; Jackson ImmunoResearch, West Grove, PA) or donkey anti-rabbit Alexa 488 (1:1000; Molecular Probes) for 1 h at room temperature followed by nuclear staining with Hoesct or DAPI. Confocal microscopy was performed using Olympus 1000 scanning confocal microscope from Olympus America at the Center for Biological Imaging, University of Pittsburgh. Final composites were constructed in Adobe Photoshop CS (Adobe, San Jose, CA).

4.8.8 Assay for Integrated HIV-1 DNA

To evaluate the integrated DNA in infected DC, infected DC (based on EGFP+/DC-SIGN+ double positive) were sorted using the MoFlo sorter at UPCI Biocontainment Flow facility with the purity of 100%. Sorted DC were evaluated for integrated HIV-1 DNA by real time Alu-LTR PCR method as described in Butler et al [110]. Briefly Cellular DNA was extracted from sorted cells using Qiagen QIAamp DNA Blood Mini Kit, and 500ng of DNA was used for PCR reaction, along with 50nM forward primer, 300nM reverse primer and 100nM of probe. The sequence of primers and probes are described in Butler et al [110].

4.8.9   Statistical analysis

The results were expressed as mean ± standard deviation. The data were analyzed using the Student's t test for paired samples. Statistical evaluation of relation between antigen uptake and DC infection was performed using linear regression analysis and R^2 value was calculated.

4.9   RESULTS

4.9.1   Effect of Vpr dysregulated T Lymphocytes on Dendritic cell

DC were generated as described in methods and were cocultured with HIV-1wt or HIV-1ΔVpr infected lymphocytes at a ratio of 2:9:1 (DC: uninfected PBL: infected PBL) either directly or separated by a transwell with pore size of 0.4μm. In some experiments, infected T cells obtained from HIV-1wt EGFP or HIV-1ΔVpr EGFP infected cultures were sorted by FACS and cocultured with DC at a ratio of 1:10 (DC: infected cell). Twenty-four hours post coculture, cells were stained for DC-SIGN, CD80, CD83, CD86 and HLA-DR; and the expression of the costimulatory molecules on DC were determined by flow cytometry. Results indicate that both in direct coculture and culture separated by transmembrane/transwell, HIV-1 infected T cells were able to upregulate the surface expression of CD80, CD83 and CD86 in cocultured DC and HIV-1 vpr did not have any significant effect in multiple donors (N=4). But we found that EGFP+ DC were present in the coculture, suggesting that the virus from cocultured infected T cells, were infecting the DC in cis.
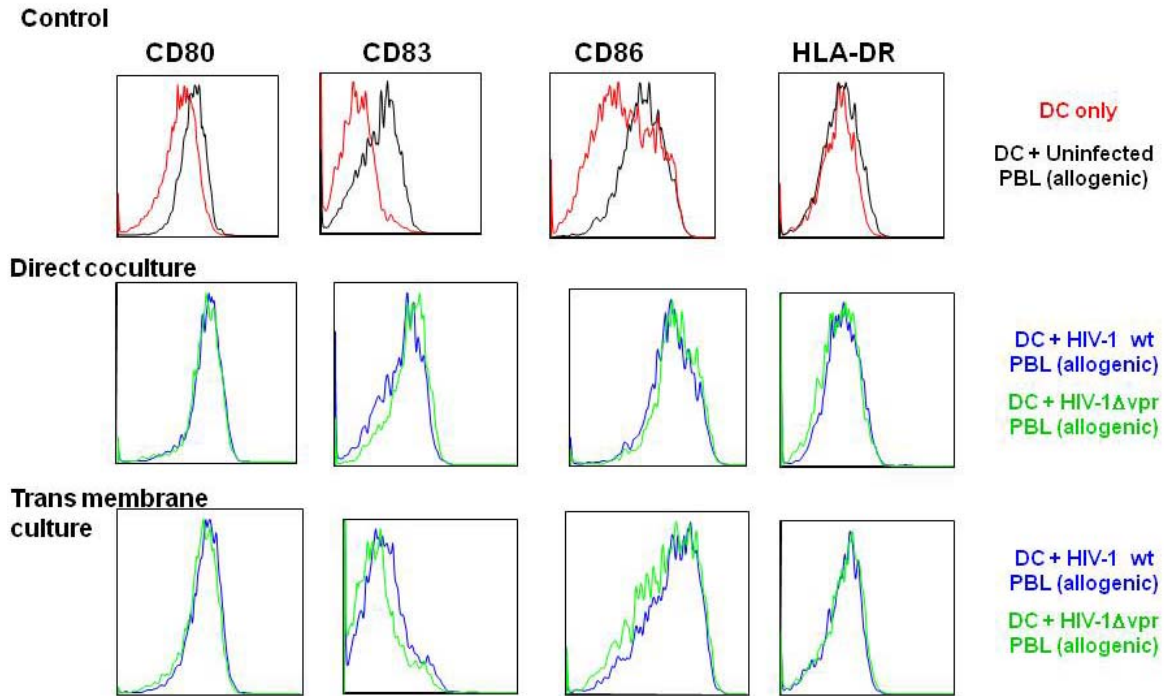
Figure 16. Effect of HIV-1 Vpr dysregulated T Lymphocytes on Dendritic cells.

DC generated as described in methods were cocultured with HIV-1wt or HIV-1ΔVpr infected T cells at a ratio of 2:9:1 (DC: uninfected PBL: infected PBL) either directly or separated by a transmembrane. Twenty-four hours post coculture, cells were stained for CD80, CD83, CD86 and HLA-DR; and the expression of the costimulatory molecules on DC were determined by flow cytometry.

4.9.2   Productive infection of immature DC by cell associated virus

Results presented above suggest that HIV-1 virus can transfer from cocultured T cell to DC and infect DC in cis. To evaluate further this phenomemon, cells were stained with DC-SIGN and analyzed by flow cytometry. DC-SIGN were gated based on side scatter and forward scatter followed by doublet discrimination gating (Fig.17A). Single cells that are double positive for DC-SIGN+ and EGFP+ were considered as productively infected DC (Fig.17A). Results from coculture experiment indicate that 7.6% of DC were infected at 12 hours post coculture with infected lymphocytes, whereas cell free virus did not infect DC (0%) at this time point (Fig.17A).
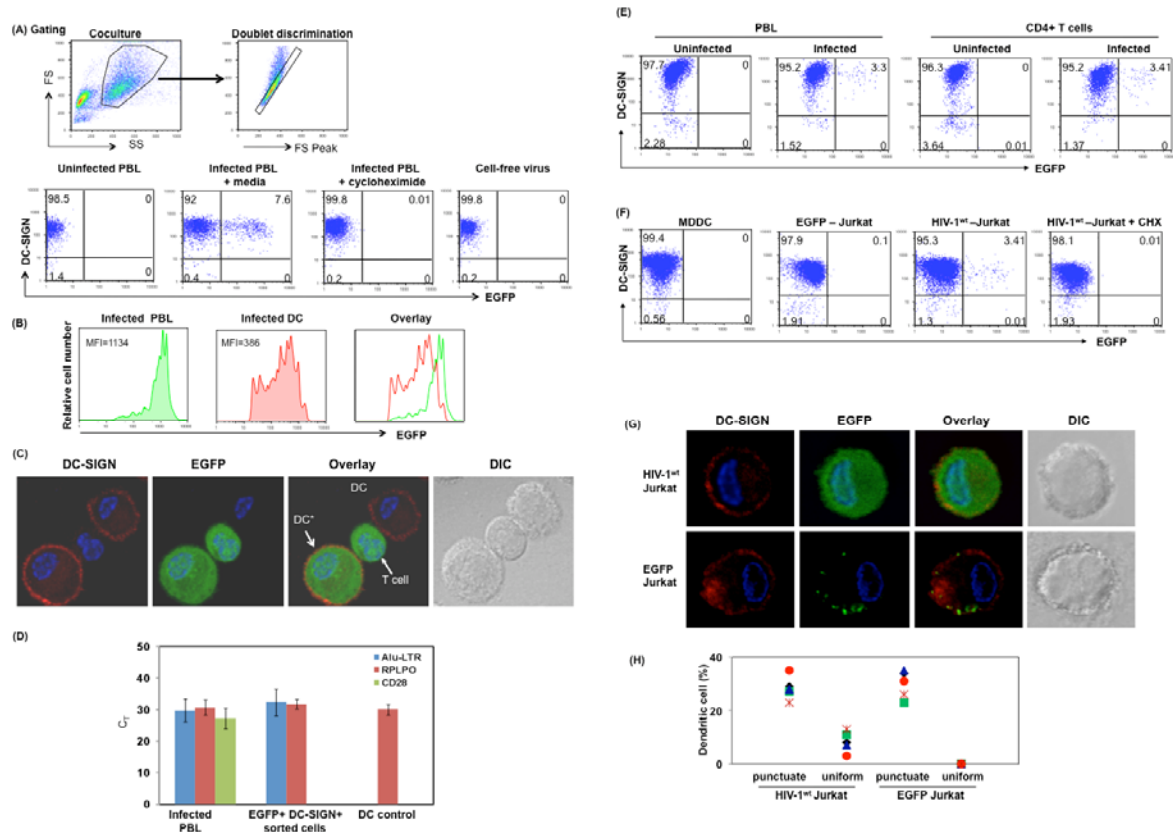
Figure 17. Reporter virus positive DC are the result of cis infection of DC.

(*A*) DC were cocultured with HIV-1^wt^-EGFP reporter virus-infected PBL cells in the presence or absence of cycloheximide (10μg/ml); or infected with cell-free virus. Post coculture (12 hrs), cells were stained for DC-SIGN. DC were gated based on side scatter and forward scatter followed by doublet discrimination (as shown in gating) and assessed for EGFP by flow cytometry. DC-SIGN and EGFP positive cells (%) are shown in the upper right quadrant. (*B*) Comparison of EGFP fluorescence (MFI) in infected lymphocytes and infected DC. Overlay of histogram of EGFP fluorescence in infected lymphocytes (green) and infected DC (red). (*C*) Detection of DC expressing EGFP by immunofluorescence microscopy. Red indicates DC-SIGN positive cells; green represents EGFP positive cells; Blue represents nuclear staining by DAPI. DC*, represents DC-SIGN and EGFP positive DC. (*D*) Detection of integrated HIV-1 proviral DNA in EGFP+ DC. DC were stained for DC-SIGN, and DC-SIGN+/EGFP+ DC were sorted by FACS. Integrated proviral DNA was assessed by real time Alu-LTR Taqman assay as described in Methods. To rule out contaminating lymphocytes in DC-SIGN+/EGFP+ sorted DC, mRNA from the sorted cells were evaluated for presence of CD28 mRNA by real-time PCR. RPLPO was used as control. Uninfected DC, Infected PBL controls were included. (*E*) DC were cocultured with HIV-1^wt^-EGFP reporter virus-infected PBL cells or with HIV-1^wt^-EGFP reporter virus-infected purified CD4+ T cells, or uninfected control cells. Twelve hours post coculture, cells were stained for DC-SIGN and assessed for EGFP positivity by flow cytometry. Cells (%) that are positive for DC-SIGN and EGFP are shown in the upper right quadrant. (*F*) DC were cocultured with either HIV-1^wt^-EGFP reporter virus-infected Jurkat T cells or with Jurkat cells expressing EGFP protein. Post coculture, the cells were stained for DC-SIGN and analyzed by flow cytometry or by (*G*) Immunofluorescence microscopy. DC-SIGN+/EGFP+ cells were gated based on the amount of EGFP in DC-SIGN+ cells to differentiate antigen uptake and productivly infected DC. Results from multiple donors are shown in Fig.17C, where 200 DC were counted in case of each culture. Figure represents one of 5–7 independent experiments with similar results.

Addition of cycloheximide (CHX) (10μg/ml) during coculture completely blocked infection of DC further confirming that EGFP expression in infected DC was due to *de novo* synthesis, and not due to cell conjugates or cell fusion. Comparison of Mean Fluorescence Intensity (MFI) of EGFP in infected DC and infected lymphocytes present in the same coculture (Fig.17B), indicates that transcription of HIV-1 LTR driven *EGFP* in infected DC is significantly less compared to infected lymphocytes. DC infection was further confirmed by fluorescence microscopy where, DC-SIGN positive cells were EGFP also positive (Fig.17C) as identified by the uniform subcellular distribution of EGFP that is indicative of *de novo* synthesized EGFP. To further validate infection in DC, integrated proviral DNA was measured in EGFP+ DC. To assess integrated proviral DNA, DC-SIGN+/EGFP+ DC were sorted and assessed for integrated proviral DNA by real time Alu-LTR Taqman assay, and for CD28 mRNA by real-time PCR. Uninfected DC and infected lymphocytes were used as negative and positive controls, respectively. Results indicate that integrated DNA was detected by Alu-LTR Taqman assay in DC-SIGN+/EGFP+ DC (Fig.17D). Additionally these cells were also negative for CD28 mRNA (Fig.17D), further confirming that integrated proviral DNA detection in sorted DC was not due to contamination of infected T cells in the culture. Together these results indicate that EGFP expression in DC is due to integrated proviral DNA that is indicative of *cis* infection. Similarly purified CD4+ T lymphocytes infected with the HIV-1^wt-EGFP reporter virus also infected DC in cell-associated manner (Fig.17E).

DC are known to take up antigens/apoptotic cells by endocytosis, micropinocytosis and other mechanisms [111, 112]. Therefore, we next delineated the uptake of cellular materials, including EGFP protein from the infected T cell versus *de novo* synthesis of EGFP in DC. DC were cocultured with either HIV-1^wt-EGFP reporter virus-infected Jurkat T cells or with Jurkat

cells expressing EGFP protein by transient transfection and assessed by flow cytometry. Results presented in Fig. 17F indicate that 3.41% DC are positive for EGFP following 12 hours of cocultured with HIV-1 infected cells. We also confirmed EGFP synthesis versus EGFP uptake in DC by fluorescence microscopy (Fig. 17G) and observed a uniform cytoplasmic and nuclear distribution of EGFP in infected DC (right panel), whereas, punctuate pattern was noted in DC following EGFP uptake (left panel). Similar results were observed in multiple donors (Fig.17H). The difference seen in the amount of EGFP in DC taking up the antigen and infected DC was not due to differences in the amount of EGFP in the cocultured Jurkat- EGFP cells or infected Jurkat cells. Together these results indicate that, EGFP+ DC seen in coculture experiment are due not to EGFP (antigen) uptake but rather it is due to *de novo* synthesis of EGFP in infected DC.

4.9.3 Productive infection of immature and mature DC is cell contact dependent
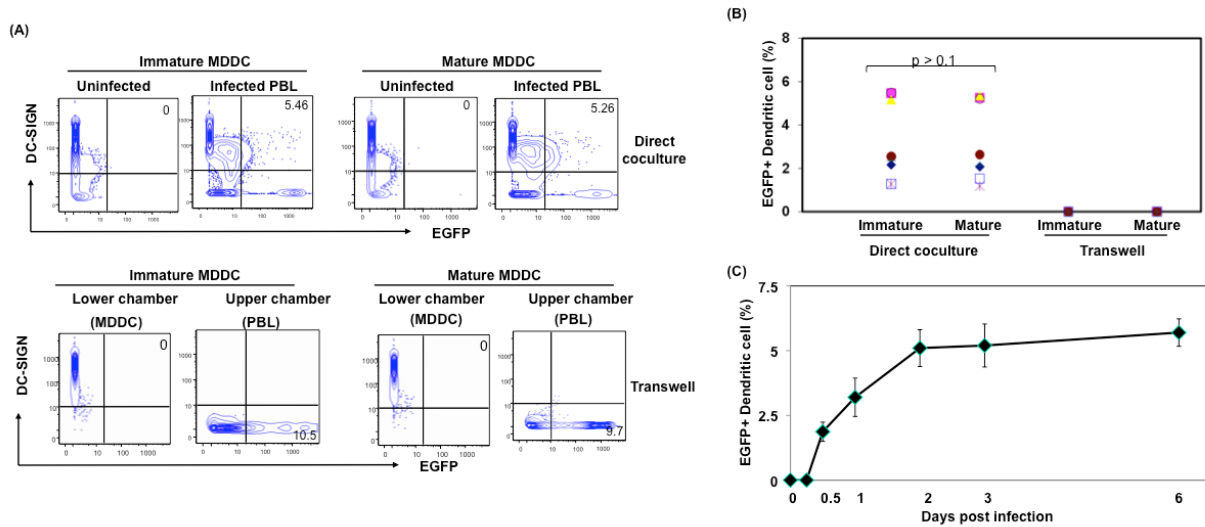
Figure 18. Infection of immature and mature DC by infected lymphocytes is cell-to-cell contact dependent.

(A) Immature and mature MDDC were cocultured with HIV-1^wt^-EGFP virus infected PBL at a ratio of 2 : 9 : 1 (DC:uninfected PBL:infected PBL) either directly or separated by a transwell. Post coculture (12hours) cells were stained for DC-SIGN, and analyzed by flow cytometry. Gating was extended to include lymphocytes and doublet differentiation was applied. (B) Cell contact dependent productive infection of immature and mature DC by

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Next, to assess whether both immature and mature DC could be infected with cell-associated virus, immature and mature DC were cocultured with infected lymphocytes as described in Fig.17. Additionally, to differentiate the role of cell free and cell-associated virus in DC infection, infected T cells were separated from DC via a transwell with a pore size of 0.4μm which will allow free virus released from infected lymphocytes in the upper chamber to pass to DC in the lower chamber, but prevent contact between infected T cells and DC. Results indicate that no EGFP+ DC (0%) when they were separated by transwell, whereas, 5.2% EGFP+DC-SIGN+ DC was observed in mixed culture (Fig. 18A). Similar results were observed in multiple donors (Fig. 18B), suggesting that cell-to-cell contact is necessary for DC infection within 12 hours. Additionally, DC from the same donors infected with cell-free virus did not show productively infected DC at the same time point (data not shown). Time course analysis indicates that DC-SIGN+/EGFP+ cells remained positive for EGFP up to 6 days post coculture (Fig.18C). Additionally, cell-free virus released from the infected T cells reaching the lower chamber established infection (<0.2%) only in immature DC 3 days post exposure (6-8 infected cells per 20 high power fields were detected by microscopy). Similar low level cell-free virus mediated DC infection was reported previously [113, 114]. These results clearly indicate that the accelerated infection of both mature and immature DC mediated by infected T cell is contact dependent and is not the consequence of cell free virus infection. Since the pore size of transwell is 0.4 m, it further rules out the involvement of exosomes derived from infected T cells in infecting DC. It is important to note that, though we observed infected DC in multiple donors, there was a wide range in percentage of infected DC (2-8%), suggesting that the variation is due

to difference in the susceptibility and/or permissibility of DC from different donors to support HIV-1 infection.

4.9.4 Infection of DC mediated by cell-associated virus does not involve DC-SIGN, Mannose Receptor, CD4 or HIV-1 envelope.

DC-SIGN and related C-type lectin receptors are suggested to play a role in *cis* and *trans* infection of DC, as blocking these receptors inhibits infection [114-116]. Therefore, we evaluated the ability of anti-DC-SIGN, anti-DC-SIGN-R antibodies, Mannan, anti-CD4 antibody, T-20 Fusion inhibitor, HIV-1 co-receptor antagonists TAK 779 and AMD 3100 to block DC infection mediated by cell-associated virus. AZT and Intergase inhibitor (118-D-24) were used as control to inhibit virus replication. Additionally cycloheximide (10μg/ml) was used as a control for *de novo* synthesis of EGFP in infected DC. As shown in Fig.19A, HIV-1 receptor and the co-receptor blockers failed to prevent infection of DC mediated by infected T cells, whereas AZT and Integrase inhibitors blocked infection by 67% and 83% respectively, compared to untreated control. Similar results were observed in multiple donors (n=5). Together, these results suggest that DC-SIGN, DC-SIGN-NR, Mannan receptors, CD4 or the HIV-1 co-receptors are not involved in T cell mediated infection of DC.
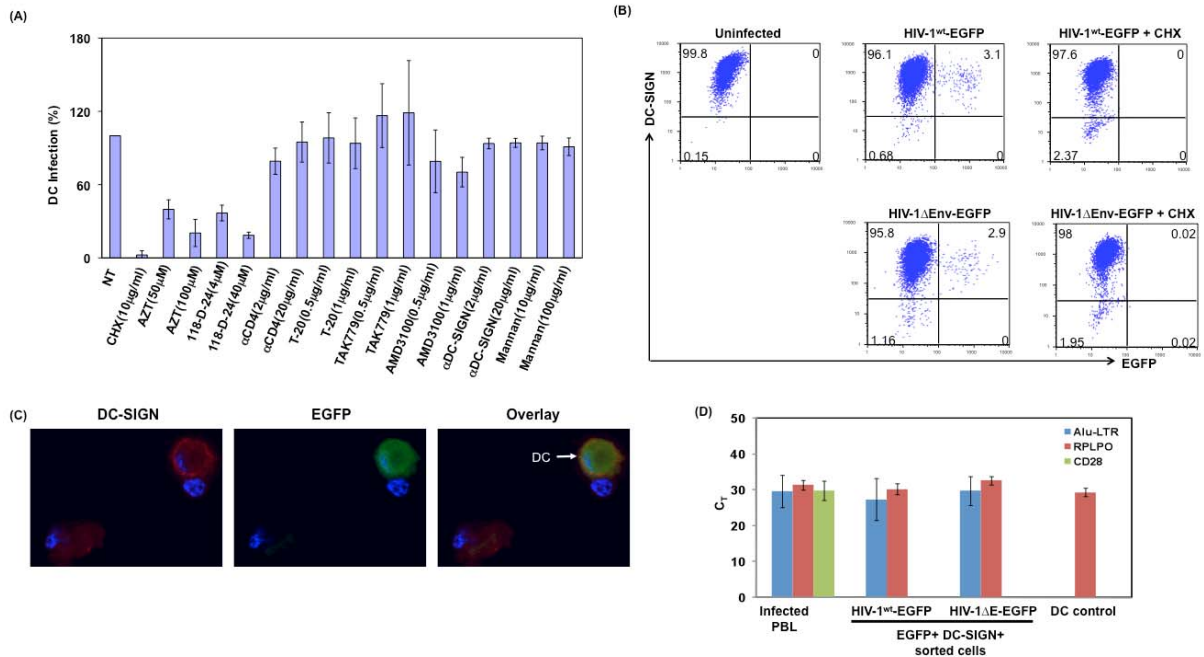
Figure 19. HIV-1 reporter virus infection of DC mediated by T cell associated virus is independent of viral envelope.

(*A*) *DC were cocultured with HIV-1^wt^-EGFP reporter virus-infected PBL cells in the presence of mentioned inhibitors. Post coculture (12 hours), cells were stained for DC-SIGN. Single DC were gated and assessed for EGFP by flow cytometry. For comparison across donors, the percentage of DC infection in absence of inhibitor was considered as 100%. Error bars indicate S.D of results obtained from results from multiple donors.(n=3) (B) CD4+ T cells were infected with HIV-1^wt^-EGFP reporter virus or with HIV-1ΔE-EGFP reporter virus complemented with vsv-G Env expression plasmid. Three days post infection cells were washed thoroughly and cocultured with immature MDDC in the presence or absence of cycloheximide (10mg/ml). Post coculture (12hours), cells were stained for DC-SIGN and analyzed by flow cytometry, and (C) Immunofluorescence microscopy. Data are representative of five independent experiments. (D) Detection of integrated HIV-1 proviral DNA in EGFP+ DC by Real Time Alu-LTR Taqman assay, following twelve hours of coculture with CD4+ T cells infected with HIV-1ΔE-EGFP reporter virus complemented with vsv-G envelope. DC were stained for DC-SIGN, and DC-SIGN+/EGFP+ DC were sorted by FACS sorter. Integrated proviral DNA was assessed by real time Alu-LTR Taqman assay as described in Methods. To rule out contaminating lymphocytes in DC-SIGN+/EGFP+ sorted DC, mRNA from the sorted cells were evaluated for presence of CD28 mRNA by real-time PCR. RPLPO was used as control.*

To assess whether presence of HIV-1 envelope is required for virus transmission from infected T cell to DC, we used HIV-1ΔEnv-EGFP virus infected T cells (pseudotyped with VSV-G envelope expression plasmid to infect T cells). Three days post infection, cells were washed thoroughly and cocultured with DC as described above and assessed for DC-SIGN+/EGFP+ cells (Fig.19B). Results indicate that DC were infected with HIV-1ΔEnv-EGFP virus as

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determined by flow cytometry in multiple donors (n=4). Flow cytometry results were validated by fluorescence microscopy (Fig.19C) and by real time Alu-LTR Taqman assay (Fig.19D). Experiments to determine the presence of contaminating lymphocytes by evaluating the presence of CD28 mRNA in the sorted, infected DC indicate no detectable CD28 mRNA, further ruled out lymphocytes contamination. Collectively, these results support that cell-associated infection of DC is independent of both DC cell surface receptors and viral envelope.

4.9.5   Infection of DC is directly correlated with the ability of DC to acquire cell-associated antigen from the interacting cell.

Results presented above indicate that transfer of virus from DC to T cell is independent of viral envelope as well as cell surface receptors in DC, suggesting that receptor independent mechanisms may be involved in virus transfer. DC are known to acquire surface molecules and cell-associated antigens from interacting cells [117, 118]. To understand whether antigen uptake mechanism is involved in virus transfer from infected T cell to DC, we examined the interrelationship between antigen uptake and DC infection. Immature and mature DC were cocultured with uninfected PKH26-labeled or infected PBL, Jurkat cells, HeLa-T4 or HEK 293T at a ratio of 1:1. DC were assessed for PKH26 uptake, and infection (EGFP+) by flow cytometry. As shown in Figure 20A, 93.8% of immature DC acquired PKH26 labeled material from PBL, 96.2% from Jurkat, 34.7% from Hela-T4 and 32.6% from HEK 293T cells. Whereas, mature DC cocultured with PKH26 stained cells efficiently acquired PKH26 labeled material from PBL (70.8%) and Jurkat cells (87.3%) but failed to acquire PKH26 labeled material efficiently from HeLa-T4 cells (6.3%) or HEK293T cells (15.4%). Results indicate that immature DC acquired membrane from tested cell types to different proportions. Although the percentage of immature

and mature DC positive for PKH26 is similar in case of PBL and Jurkat, it is important to note that the amount of PKH26 acquired by mature DC was lower than immature DC as seen by the MFI (Fig. 20B). These results indicate that both immature and mature DC uptake antigen from T cell lineage more efficiently and equally, whereas they exhibit differential ability to uptake antigen from epithelial cells.
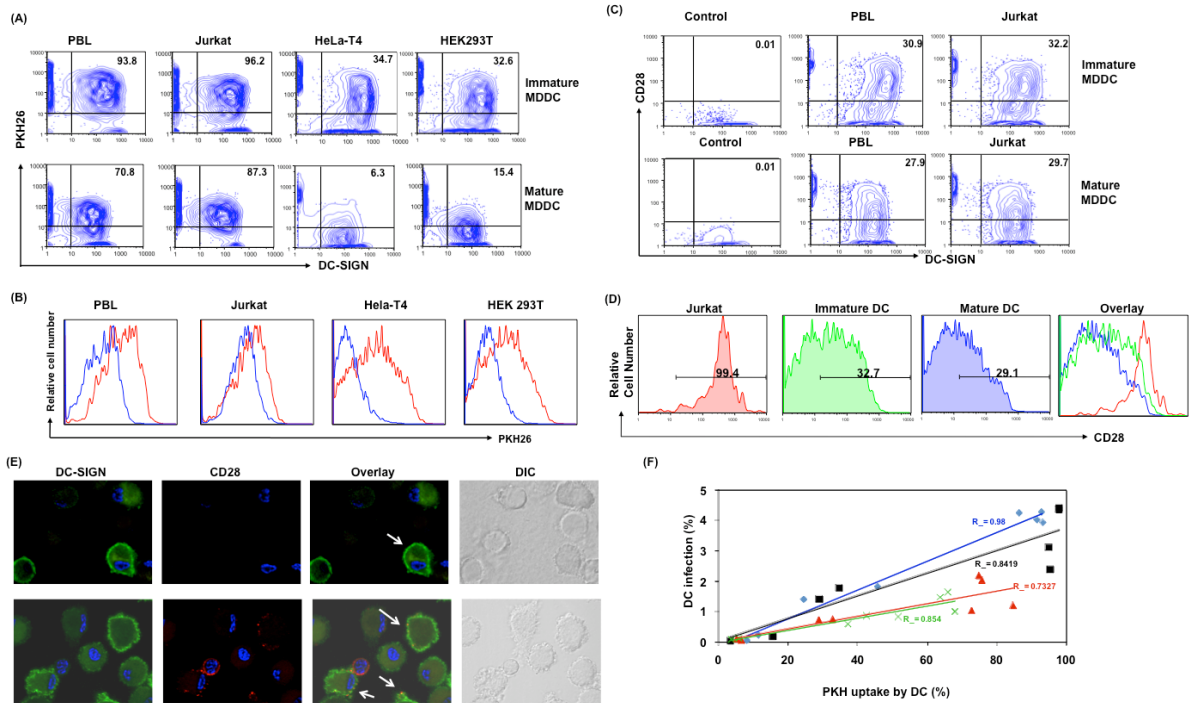
Figure 20. Infected T cell mediated DC infection is directly correlated with the ability of DC to acquire antigen from T cell.

(A) Immature and mature MDDC were cocultured with PKH26-labeled PBL, Jurkat cells, HeLa-T4 or HEK 293T at a ratio of 1:1. Post coculture (12 hours) cells were stained for DC-SIGN and the amount of PKH26 uptake by DC-SIGN positive cells were assessed by flow cytometry. DC were gated based on side scatter and forward scatter followed by doublet discrimination. Values in upper right quadrant indicate the percentage of DC-SIGN and PKH26 positive cells. (B) Histogram overlay represent PKH26 fluorescence in immature (Red) and mature (Blue) DC, post cocultured (12 hours) with PKH-26 labeled PBL, Jurkat cells, HeLa-T4 or HEK 293T cells. The figure is representative of data obtained from experiments in four separate donors. (C) Immature and mature DC cocultured (for 12 hours) with PBL or Jurkat T cells were stained for DC-SIGN and CD28 and evaluated by flow cytometry. (D) Comparison of MFI of CD28 molecule on the surface of Jurkat, immature DC or mature DC cocultured with Jurkat T cells. (E) Immature DC were cocultured with Jurkat T cells for twelve hours, cells were stained for DC-SIGN and CD28 and evaluated by Immunofluorescence microscopy, green represents DC-SIGN, CD28 is depicted in red and DAPI staining of nucleus is shown in blue. DC alone control was included. (F) Scatter plot denotes the correlation of PKH26 uptake and DC infection post coculture with different cell types. Each color in the plot denotes individual donor (n=4). Linear regression was calculated for each donor, along with the R^2 value.

In addition to cytoplasmic and membrane bound antigen uptake, APC are known to acquire membrane from the interacting cell surface on to their own surface in the right orientation, a phenomenon described as trogocytosis [119-121]. To understand whether DC infection is mediated through this mechanism, we evaluated the ability of DC to acquire T cell surface molecules, CD3 and CD28, by flow cytometry. We performed surface staining of these molecules using CD3 or CD28 specific antibodies, which will specifically detect these molecules, if they orient on the outer side of the membrane via trogocytosis. Results indicate that both immature and mature DC acquired CD28 from the interacting PBL or Jurkat. This observation was further confirmed by confocal microscopy by staining DC with anti-CD28 antibody. Results indicate that the presence of CD28 on DC cell surface, where speckles of CD28 was identified (Fig. 20E). Together these results indicate that DC acquire membrane from their interacting T cells via antigen uptake and trogocytosis.

When we compared the infection of DC within these cultures, we also observed that there is a direct correlation between membrane uptake and infection. In multiple donors (n=4), at 12 hours following coculture at a ratio of 1:1 (DC: PBL) (10% of PBL are infected) it was observed that both immature and mature DC were infected at the highest in case of PBL (2.7$\pm$1.1) and Jurkat (2.6$\pm$1.8) coculture, whereas, it was almost half when immature DC were cocultured with HEK293T (1.3$\pm$0.54) and HeLa-T4 (1.1$\pm$0.35) cells. Interestingly, mature DC did not show any infection when cocultured with HEK293T (0.16$\pm$0.07) or HeLa-T4 (0.08$\pm$0.04) cells and is directly correlated with the low/no antigen uptake. Statistical evaluation between PKH26 uptake by immature and mature DC from different cell type (PBL, Jurkat, Hela-T4, HEK 293T) and associated DC infection in multiple donors (n=4), indicates a direct correlation between antigen uptake and infection of DC, R^2 value ranges from 0.98 to 0.73 (Fig. 20F). Together these results

support that there is a strong correlation between antigen uptake and infection of DC via T cell-associated virus.

4.9.6 Blocking the ability of DC to acquire cell-associated antigen prevents DC infection.

As the ability of DC to acquire cell associated antigen and infection of DC is directly correlated, we further investigated whether blocking the membrane uptake will result in loss of DC infection. DC were cocultured with infected PBL in the presence and absence of cytochalasin D, Colchicine, AZT and evaluated for membrane uptake as well as DC infection (Fig.21A). Results indicate that cytochalasin D blocked both membrane uptake and infection (>80%) significantly, whereas colchicine did not show any effect of the membrane uptake or infection compared to the no treatment group. In case of AZT, it did not affect membrane uptake, whereas, it inhibited infection in DC by 75-80% at 100µM compared to untreated group.

Furthermore, results using various concentrations of cyctochalasin D indicate that cytochalasin D inhibited membrane uptake as well as DC infection in a dose dependent manner (Fig.21B). At a concentration of 0.1µg/ml, the antigen uptake was reduced by $67.6\pm4.7\%$ and the infection was inhibited by $71.1\pm5.1\%$, where as, at 1µg/ml, the antigen uptake was reduced by $81.3\pm5.7\%$ and the infection was inhibited by $87.3\pm3.8\%$. Cytochalasin D at concentrations of 5 and 10µg/ml inhibited the antigen uptake to more than 90% and at these concentrations there was complete inhibition of infection in DC (Fig.21B). Inhibition of infection was independent of cytotoxicity induced by cyctochalasin D as confirmed by annexin V staining in DC (data not shown). Together these results indicate that DC might acquire virus from infected T cells through the antigen/cell membrane uptake mechanisms.
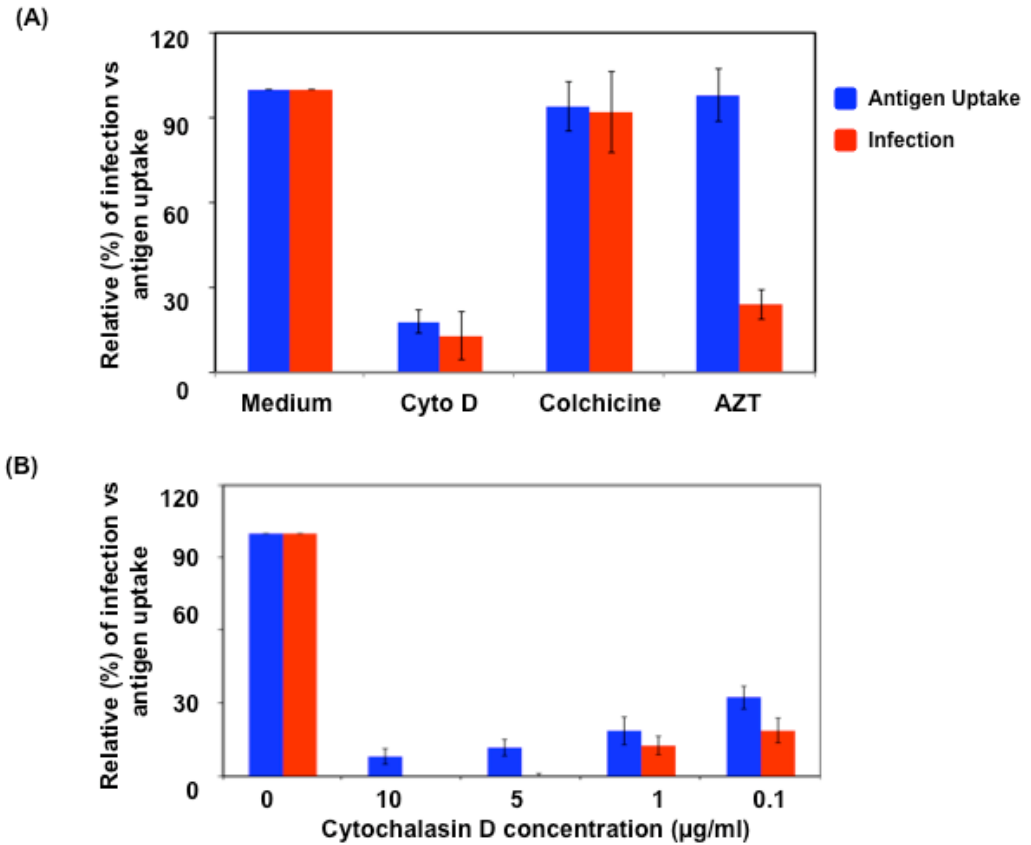
Figure 21. Blocking the ability of DC to acquire cell associated antigens prevents DC infection by T cell associated virus.

(A) DC were cocultured with infected T cells in the presence of various inhibitors (Cytochalasin D 1µg/ml; Colchicine 100 µg/ml; AZT 100µM),post coculture (12 hours) were stained for DC-SIGN and the amount of antigen uptake by DC and percentage of productive infection of DC were evaluated by flow cytometry. The figure is representative of data obtained from one of the five independent donors. Error bars indicate S.D. of the results obtained from triplicate wells from a single donor. (B) Ability of Cytochalasin D to inhibit antigen uptake and productive infection of DC in dose dependent manner. The figure is representative of data obtained from one of four independent experiments.

4.10   DISCUSSION

Both cell-free and cell-associated virus facilitates transmission, spread and dissemination of HIV-1. However, cell-associated virus infection has added advantages and is more effective than cell-free virus infection. Viral dissemination through cell-to cell contact is mediated through virological synapses, and this event is predominant at the secondary lymphoid organs [122, 123]. Several host cellular proteins, such as ICAM, LFA, ZAP-70 are known to regulate this event [124-128]. One of the ways these cellular proteins regulate virus transmission is through enhancing cell-to cell contact, ability these proteins to incorporate in the virus particles, suggesting that viruses utilize several modes for efficient transmission. Published studies also indicate that antiviral drugs, AZT or neutralizing antibodies do not block the cell-associated HIV transfer and infection [129, 130]. Together these findings suggest that virus transmission also occurs through other mechanism(s) that are not well established.

In this study, we have shown for the first time that DC acquire virus from infected T cells utilizing the antigen uptake mechanisms that results in DC infection. Previous studies have focused on DC handing off virus to the interacting naïve T cells as part of the "trojan horse" model that results in productive infection [114, 131]. However, it is not well understood whether a reverse phenomenon is possible. This is important as DC interact with infected T cells to sample foreign antigens for priming naïve T cells. Our results indicate that DC acquire virus from the infected cells during the antigen uptake process that results in DC infection. Although immature DC efficiently uptake antigen (>90% of total DC cocultured) from T cells, infection of DC is 5-8% of the total DC cocultured, suggesting that a small amount of virus is able to escape the antigen processing pathway and establish infection. A recent study by Turville et al [132], further support our finding that DC can take up virus from another infected DC.

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ANTIGEN UPTAKE
ICAM : LFA /TALIN
MHC CLASS II : TCR
CD28/CTLA-4 : CD80/CD86
CD40:CD40L
OTHER MOLECULES / FACTORS ?

TRANSFER OF
VIRUS PARTICLE,
PROVIRAL DNA
MOLECULES / FACTORS ?

Non-Integrated
Proviral DNA

Integrated
Proviral DNA

Budding Virus
particle

DC

Uninfected T Lymphocytes

Infected T Lymphocytes
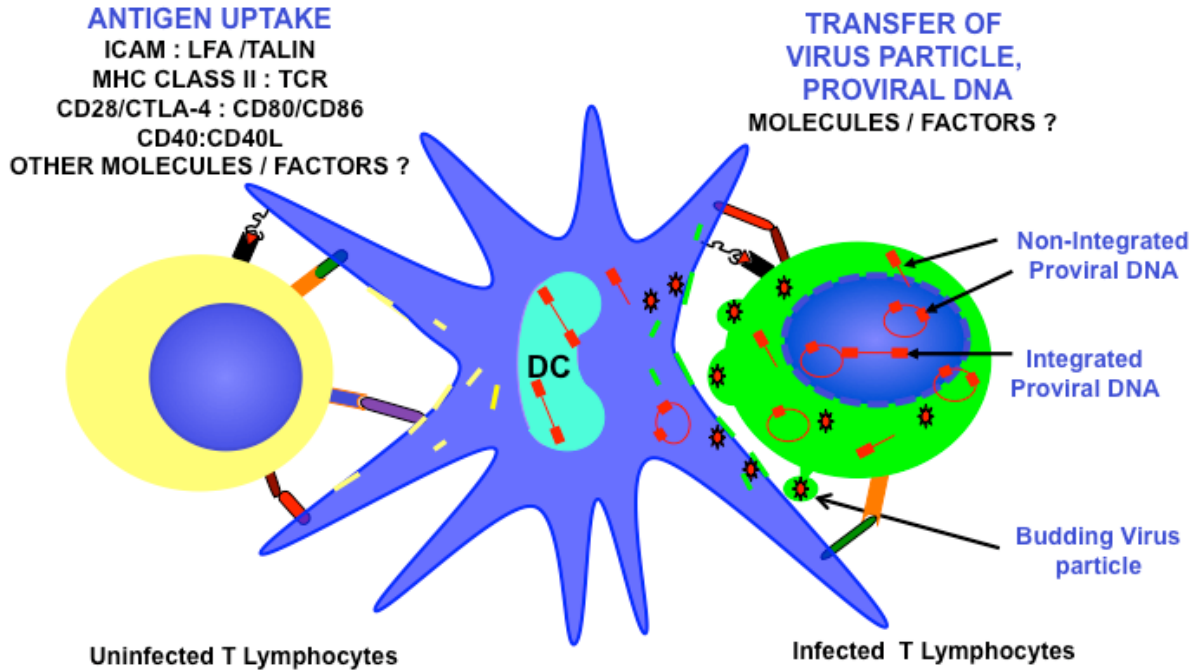
Figure 22. Proposed model depicting the various mechanisms(s) involved in virus transfer from infected T cell to DC.

This phenomenon might have a significant impact *in vivo*, as DC and T cells interact at multiple sites including the site of entry (mucosal tissue) and lymphoid structures within the infected host. Upon infection by pathogens, various immune cells (infected and bystander) come in contact at the lymphoid tissues for antigen uptake, presentation, priming and induction of immune responses. Many of these processes occur through the formation of immunological synapses [133-135]. Pathogens, including HIV-1 are known to dysregulate immunological synapse and enhances virological synapse by differentially regulating viral and host cellular factors [122, 136, 137].   Utilizing this immunological process, DC capture free virus and efficiently *trans infect* T cells *in vivo* and *in vitro*. *In vivo* studies (animal model) indicate the presence of productively infected T cells 3 days post-intravaginal inoculation [138, 139]. Here

we address the consequence of the interaction between an infected T cell and uninfected DC and its potential role in viral dissemination.

Results presented here indicate that DC acquire virus from infected T cells independent of cellular and viral receptors that are involved in typical cell-free infections but dependent of cell-to-cell contact, suggesting that cell-to-cell communication and/or cellular networks are involved in virus transfer. Although results presented above indicate that DC might acquire virus from infected cells, via their antigen uptake mechanisms, it is not clear what kind of material transfer occurs between T cell and DC. Based on the cell surface ligands, their receptors involved in DC-T cell interaction, it is possible to predict that the presence of these costimulatory molecules might increase the affinity of cell-to cell contact though their ligands present in DC. Based on the available information, we proposed several scenarios that could be the source for DC infection as shown in Fig. 22. These include: (a) uptake of budding virus particle from the infected T cell via cell membrane uptake; (b) uptake of various forms of infectious unintegrated viral DNA from infected T cell cytoplasm via cytoplasmic antigen uptake; (c) membrane transfer of assembling and budding virus from the infected T cell and reorient on DC membrane; and (d) uptake of cellular and nuclear content including viral antigens and viral nucleic acids from the apoptotic infected T cells. Alternatively, virus could also transfer from cell to cell via a cellular network including nanotubules, and other related extensions, as DC acquire dyes, bacteria and other pathogens from cells through nanotubules [111, 140]. Transfer of viral nuclear material, either unintegrated viral DNA as LTR circles or linear proviral DNA may have an effect in viral pathogenesis. It should be noted that these unintegrated forms have varied half life, the linear unintegrated proviral does not survive for long, but conflicting reports suggest that the LTR ring forms may persist in the cells and may have a role in virus persistence, even in patients on

HAART for a long duration. Alternatively DC can acquire immature virus particle, which may not be infectious. *In vivo,* DC scavenges the tissues for foreign antigens as part of normal immune surveillance. If HIV-1 utilizes these normal DC cell functions for virus transmission, this will have significant impact on pathogenesis and disease progression. Clearance of virus from the infected host will be much more difficult. Understanding the mechanism(s) involved in contact dependent virus transfer will further enhance our knowledge towards developing additional antiviral strategies to prevent HIV-1 transmission.

5.0 STRUCTURE-FUNCTION ANALYSIS OF HIV-1 VPR IN IMMUNOPATHOGENESIS, VIRUS REPLICATION AND DISEASE PROGRESSION: FOCUS ON VPR OLIGOMERIZATION AND FUNCTIONS.

5.1 INTRODUCTION

HIV-1 Vpr, a non-structural protein, is incorporated in the virus particles and possesses several characteristic features that are known to play important roles in HIV-1 replication and disease progression. Vpr has a positive role in efficient transport of PIC into the nucleus of non-dividing cells and enhance virus replication in primary T cells [141-144]. Vpr also has a well-defined role in apoptosis, cell cycle arrest and dysregulation of immune functions [20, 31, 145]. Many of the Vpr functions are carried out by virion-associated Vpr similar to *de novo* synthesized Vpr, suggesting that incorporation of Vpr into virus particles is an important event in HIV-1 biology. Biochemical analysis and NMR studies suggests that Vpr has three α-helix connected by loops, and interacts with each other to form oligomers [146-149]. Single residue based site directed Vpr mutagenesis suggests that amino acids in the N terminal region are essential for stability, amino acids in Helix II are essential for virion incorporation and region in the Helix III and C terminal region determine the nuclear transport of Vpr [150-153]. However, structure-function studies using biologically relevant Vpr alleles derived from HIV-1 patients are not available.

To gain a better understanding of Vpr's role in pathogenesis *in vivo*, we compared primary aminoacid sequences of Vpr alleles inform a well defined Long Term Non-progressors (LTNP) and Rapid Progressors (RP) population. This was compiled by combining information available from Los Alamos Database and GenBank. Results from these studies indicate that though Vpr alleles have variable nucleotide and aminoacid sequences, the alleles tend to maintain lesser degree of variability with respect to polarity and charge. There is comparatively more variability in the hydrophobicity index, in the LTNP group than RP, suggesting a role for the structure of Vpr in pathogenesis.

HIV-1 Vpr is known to oligomerize both *in vitro* and *in vivo* [148, 154]. This has been demonstrated using Vpr expressed in cells, in the context of transfection of plasmid DNAs and through virus infection. Similar observations have also been reported with the purified Vpr protein from the prokaryotic expression system. Vpr has been shown to exist as dimers. trimers, tetramers and multimers [148]. In general, protein oligomerization is thought to be an advantageous feature for reasons of increased stability, interaction/binding with other proteins, allosteric control and the establishment of higher-order complexity [155]. Vpr is known to interact with viral and cellular proteins and this interaction is essential for several Vpr mediated functions. For instance, Vpr interacts with Gag-p6 and packages in the virus particles and virion-incorporated Vpr is known to positively regulate infection of non-dividing cells and enhance virus production in T cells [156-158]. Despite this, it is not clear whether oligomerization of Vpr is required for virion incorporation and/or for its interaction with cellular proteins.

To gain a better understanding of Vpr oligomerization and its role in Vpr-induced viral and cellular functions, we have utilized a chimeric protein strategy in which HIV-1 Vpr is fused to either N- or C-terminus fragment of Venus protein. Upon expression and formation of dimers

in live cells the venus will emit fluorescence that can be detected by microscopy and/or flow cytometry. Such an approach has allowed us to evaluate the domains/residues essential for Vpr oligomerization and its relevance to Vpr functions using Bimolecular Fluorescence complementation (BiFC) analysis. Results from these studies indicate that Vpr molecules with distinct mutations in helical domains I, II and III dysregulate Vpr oligomerization, virion incorporation and Vpr-mediated cellular events.

5.2   MATERIALS AND METHODS

5.2.1   Cell culture and Plasmids

HeLa, and HEK293T cells were grown in DMEM supplemented with 10% FCS, 1% glutamine and 1% penicillin-streptomycin. Proviral construct pNL4-3^{wt}, pNL4-3ΔR, pNL4-3ΔR-EGFP were used in the studies. pNL43ΔR-EGFP was constructed as mentioned previously [109]. pNL43^{wt} was obtained from NIH ARRRP, contributed by Dr. Landau and pNL43ΔR was a kind gift from David N. Levy, University of Alabama. Vpr expression plasmids were generated using the methods as described [150]. All the mutant constructs were sequenced to verify the integrity of the mutations. For BiFC assays, sequences encoding the amino (residues 1 to 173, VN) or carboxyl (residues 155 to 238, VC) fragments of Venus fluorescence protein (template generously provided by Dr. Ronald Montelaro, University of Pittsburgh) were fused to the N terminus of HIV-1 Vpr via a six-alanine linker. All plasmids were isolated using QIAGEN Maxiprep kit (QIAGEN, Valencia, CA),

5.2.2   Western blotting

HEK293T cells were cotransfected with Vpr expression plasmid using Lipofectamine. Forty-eight hours post-transfection, cells were washed twice with PBS and lysed in RIPA buffer containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton X-100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1.0mM phenylmethylsulfonyl fluoride, 0.05% deoxycholate, 10% sodium dodecyl sulfate, aprotinin (0.07 trypsin inhibitor unit/ml), and the protease inhibitors leupeptin, chymostatin, and pepstatin (1μg/ml; Sigma). Cell lysates were clarified by centrifugation, and total cell lysates (50μg) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis gel, transferred, and immunoblotted with anti-HIV-1 p24 for Gag and anti-Vpr antibody or anti-HA for Vpr. The blots were developed using an ECL kit (Amersham Biosciences, Piscataway, NJ).

5.2.3   Immunofluorescence

Thirty-six hours post-transfection, cells were washed with PBS and fixed in 3.7% formaldehyde at room temperature for 10 minutes. Following three washes with PBS the cells were permeabilized with 0.5% Triton X-100 for an additional 10 minutes. After washing 3 times with PBS, the cells were blocked with 1% BSA at room temperature for 1 hour followed by incubation with primary antibody (HA or Vpr; 1:200 dilution, BD Biosciences) for 1 hour at room temperature and incubated with rabbit anti-mouse or anti-rabbit IgG Rhodamine (RRX) (1:400; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. Cells were mounted with VECTASHIELD mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence analysis was performed using a fluorescence microscope

with Nikon SPOT camera (Fryer, Huntley, IL) and images were processed using MetaMorph software (Universal Imaging Corporation, Downington, PA).

5.2.4   Luciferase assay

HeLa cells were transfected with HIV-1 LTR Luciferase or NF-kB-Luciferase reporter plasmid (1µg) in the presence and absence of Vpr^{wt} or Vpr mutants (0.5µg) and pCMV b-Gal using Lipofectamine (InVitrogen, CA). Forty-eight hours post transfection, cells were lysed in 500µl of 1X reporter lysis buffer and luciferase activity was measured following the manufacturer's protocol (Promega, WI). Transfection efficiency was normalized by transfecting with CMV β-gal plasmid and analyzing the β-gal activity (Promega, WI). In some experiments pEGFP plasmid was used as a control to normalize for transfection efficiency To measure NF-kB activity cells were stimulated with human recombinant TNF-a (1 ng/ml) for 3 hours before lysis to activate the reporter.

5.2.5   Annexin V staining

HeLa cells were transfected with 2.5µg of Vpr mutant molecules or vector DNA using lipofectamine as per the manufacturer's instructions. Forty-eight hours post transfection, cells were washed twice with cold FACS buffer. To detect apoptosis, cells were resuspended in 100µL sterile binding buffer containing 10mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5mM CaCl$_2$, incubated with Annexin V-FITC (BD Bio-Science) for 15 minutes at RT in the dark and diluted four times with binding buffer before analyzing by flow cytometry as described [159]. The percentage of Annexin-V positive populations was evaluated by FlowJo software.

5.3 RESULTS

5.3.1 Construction and characterization of Vpr plasmids for oligomerization studies using BiFC analysis

The ability of Vpr to oligomerize was proven by biochemical methods using bacterially produced or chemically synthesized Vpr protein and/or peptides [156]. A recent study has shown that Vpr forms dimers and oligomers in relevant eukaryotic cells by using fluorescence spectroscopy and imaging analysis [154]. These observations have prompted us to evaluate the requirement of specific domains/residues in Vpr oligomerization. It is important to note that, though investigators reported oligomerization in live cells, the assays used for this purpose are of qualitative in nature. On the other hand, dimerization between two molecules is amenable for precise analysis and is considered by several investigators as an integral part of oligomerization. Considering this, we have selected Bimolecular Fluorescence Complementation system based on Venus as a reporter protein. The chimeric proteins containing Vpr and N- or C- terminus fragments of the reporter due to the interaction Vpr are likely to result in the reconstitution of a functional Venus with fluorescence. Briefly, Vpr from NL4-3 (will be referred as Vpr^{wt}) was cloned downstream of N terminus (1-173 aa) or C terminus (155-238) of Venus fluorescent protein as described[160] and verified by sequence analysis. The schematic representation of the constructs is presented in Fig. 23A. The recombinant plasmid constructs were assessed for expression of the correctly sized protein products by transient transfection in HEK293T cells. The analysis of the lysate from transfected cells was carried out by immunoblot assay. The results shown in Fig.23B indicate that a chimeric (Venus-Vpr) protein was expressed in cells transfected with the plasmids. The chimeric protein of expected size was detected by antibodies

against Vpr and HA tag. The steady state expression levels of chimeric proteins were similar to that of wild type Vpr. Next, we also analyzed the subcellular localization pattern of the chimeric proteins in comparison to the untagged wild type Vpr to confirm that fusion of N- and C-terminal fragment of Venus reporter did not alter Vpr localization (Fig. 23C). Results indicate that VN-Vpr and VC-Vpr exhibit nuclear localization pattern, similar to wild type Vpr protein. Finally, the functional ability of these chimeric constructs to induce apoptosis (a hallmark function of vpr) was assessed by flow cytometry (Fig. 23D). Both chimeric constructs and untagged Vpr construct induced comparable level of apoptosis (51 to 56% in transiently transfected HeLa cells compared to untransfected or vector transfected control (15%). Together these results indicate that fusion of these chimeric molecules (VC and VN) did not alter the expression, localization and/or functions of Vpr.

HIV-1 Vpr interacts with Gag specifically and incorporates into virus particles[156, 161]. Therefore, these plasmids were cotransfected with pNL43Δvpr-EGFP proviral plasmid and assessed for their ability to incorporate in virus particles. The results indicate that fusion of Venus-C or Venus-N fragments with Vpr^{wt} did not alter the ability of Vpr to package in virus particles (Fig. 23E). The reactivities of chimeric Vpr to antibodies against Vpr and its incorporation into virus particles suggest that these constructs serve as a useful tool to study Vpr dimerization/oligomerization in live cells.
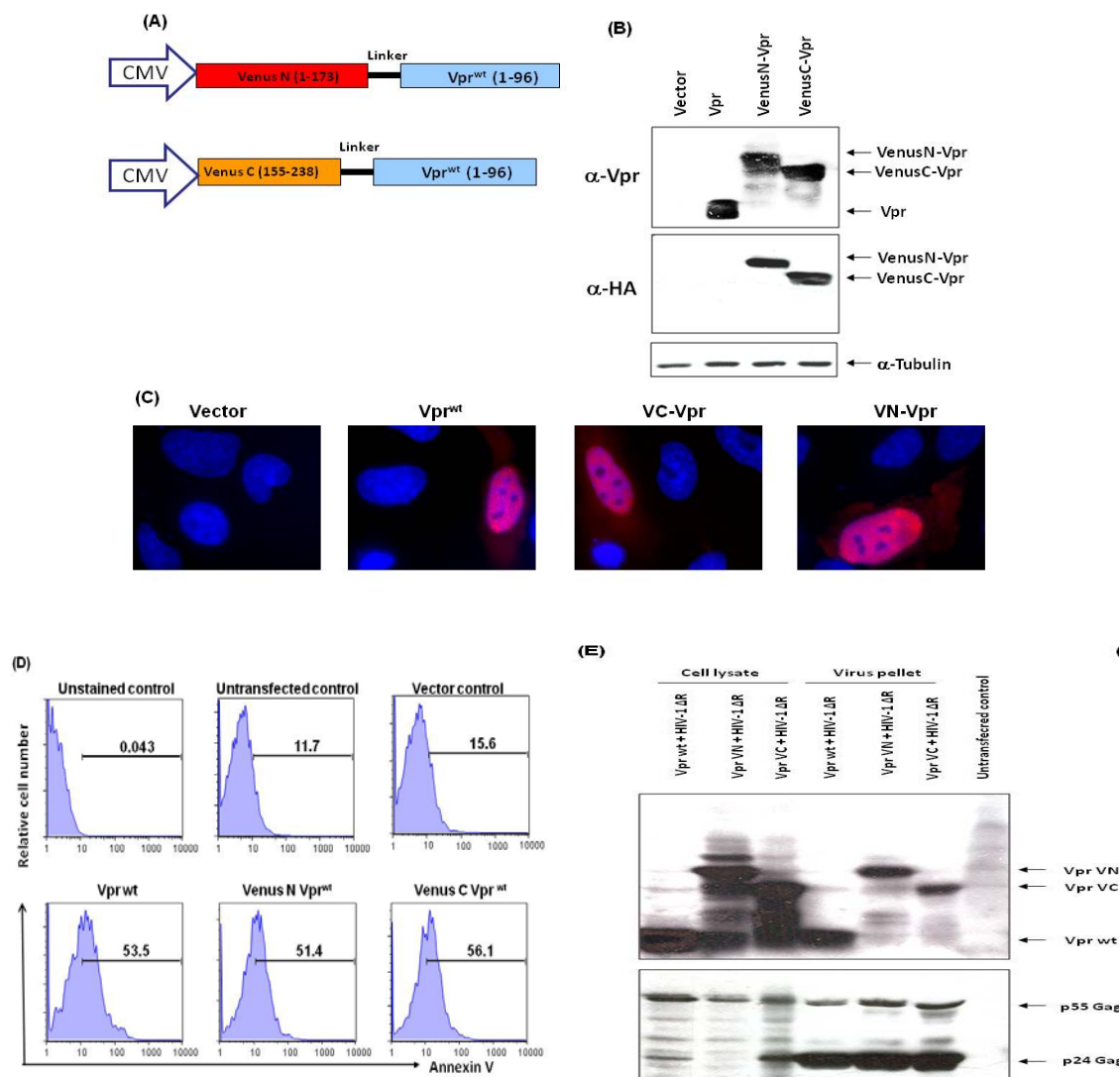
Figure 23. Construction and characterization of Vpr plasmids for oligomerization studies in live cells using BiFC.

(A) Schematic representation of Vpr^{wt} fused with Venus-N terminal or Venus-C-terminal fragments. (B) Expression of Venus-C Vpr^{wt} and Venus-N Vpr^{wt} was assessed in HEK293T cells by transient transfection. HEK293T cells were transfected with Venus-N – Vpr^{wt} or Venus C – Vpr^{wt} expression plasmids or Vector control plasmid, and assessed by Western blot. (C) Subcellular localization pattern of Vpr^{wt} or Venus-Vpr^{wt} fusion proteins was assessed in Hela cells by transient transfection. HeLa cells were transfected with Vpr^{wt} or Venus C – Vpr^{wt} or Venus N – Vpr^{wt} expression plasmids or Vector control plasmid, and assessed by Immunofluorescence for subcellular localization pattern. (D) Evaluation of Venus- Vpr^{wt} fusion proteins to induce apoptosis in HEK293T cells. HEK293T cells were transfected with Vpr^{wt} or Venus-N-Vpr^{wt} or Venus C-Vpr^{wt} expression plasmids or Vector control plasmid, forty-eight hours post transfection the cells were assessed for apoptosis by flow cytometry following Annexin V staining. Cells (%) positive for Annexin-V is marked. (E) Virion incorporation of Venus-C-Vpr^{wt} and Venus-N- Vpr^{wt} was assessed in HEK293T cells by transient transfection. HEK293T cells were cotransfected with pNL43Δvpr-EGFP proviral plasmid and Venus- Vpr^{wt} chimera or Vpr^{wt} expression plasmid, and assessed by Western blot for Vpr expression in cell lysate and virus particle. Gag was assessed as a loading control. Figure represents one of five independent experiments (n=5) with similar results.

To monitor dimerization/oligomerization in live cells, HEK293T were cotransfected with equal amount of Venus-C Vpr and Venus–N Vpr plasmids or each plasmid with background control vector. Vpr oligomerization was monitored forty-eight hrs post transfection by flow cytometry (Fig. 24A) and by fluorescence microscope (Fig. 24B). Results indicate that cells transfected with both VC-Vpr and VN-Vpr exhibit positive signal (26% of BiFC positive cells) that is detected by flow cytometry and by fluorescence microscopy, whereas VC-Vpr or VN-Vpr with a vector control plasmid did not show any signal. Similar results were observed in Jurkat cells transfected with the BiFC plasmids (data not shown). To ascertain the specificity of Vpr-Vpr interactions, we have also carried out a competition experiment in which untagged Vpr is expressed along with chimeric VC-Vpr and VN-Vpr. As expected, the inclusion of untagged Vpr has resulted in a diminished BiFC signal (data not shown). Together these results indicate that oligomerization of Vpr is specific and this technique would allow us to study the interaction in a live cell more efficiently, especially in HIV-1 target cells.
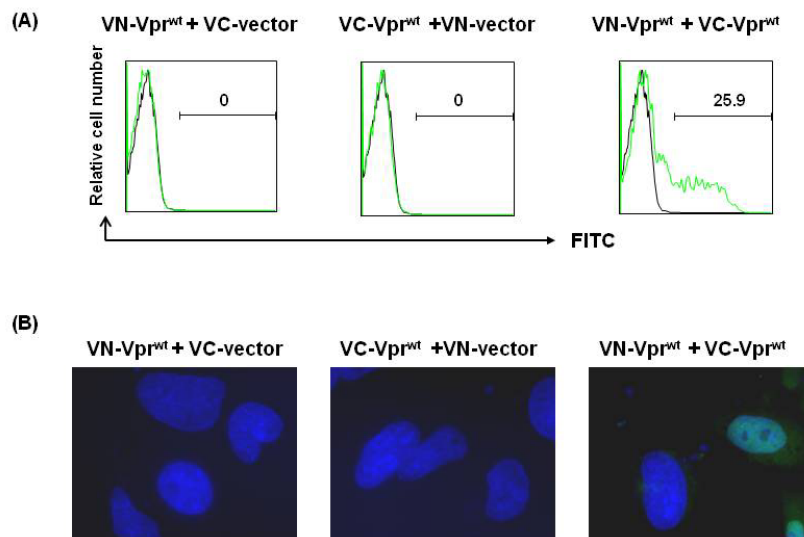
Figure 24. Visualization of Vpr dimerization in live cells.

(A) Quantitative analysis by flow cytometry of Venus fragment complementation in HeLa cells transfected with VC-Vpr and VN-Vpr or with control plasmid. Thirty-six hours posttransfection, cells were harvested and analyzed by flow cytometry to determine the percentage of cells positive for BiFC fluorescence. Results represent the means of five independent experiments. (B) Subcellular localization of the BiFC complex. HeLa cells grown on

5.3.2 Comparison of Vpr alleles obtained from Rapid Progressors and Long Term Non Progressors.

Sequences of Vpr alleles (N=1223) from Los Alamos National Laboratory Database were considered for the study, these included Vpr sequences, which started with Methionine and had around 96 amino acids; truncated Vpr sequences were eliminated from the analysis. Further information regarding the association of the Vpr allele with the progression of the disease was obtained from GenBank Database. Compiling both these database together, we were able to identify sequences of Vpr alleles derived from rapid progressor (N=102) and long term non progressors (N=193). Status regarding the progression of disease was not known for the remaining sequences. HIV-1 Vpr has three helices, Helix I (17-33), Helix II (38-50), Helix III (54-77), which are reported to play a critical role in maintaining the structure-function of Vpr. Comparison of data across these two groups indicate that most of the residues in the helices are significantly conserved. Variations are observed in certain residues in both RP and LTNP population. In most cases of variations, there is preservation of polarity and charge of aminoacid (example – 28,32,41, 48, 60, 68). We also found a some residues were significantly different in LTNP and RP (19,48,77), suggesting the importance of structure-function of Vpr, hence long term nonprogression status, examples include mutation in Vpr at 48 position - E48A, this mutation results in loss of vpr incorporation in to virion particle; Mutation at 77 – R77Q results in loss of Vpr property to induce apoptosis. These results suggest that, the fitness pressure on the

virus tends to conserve the Vpr at the structural perspective, and any adverse mutation in structure-function of Vpr favorably affect the progression of the disease.
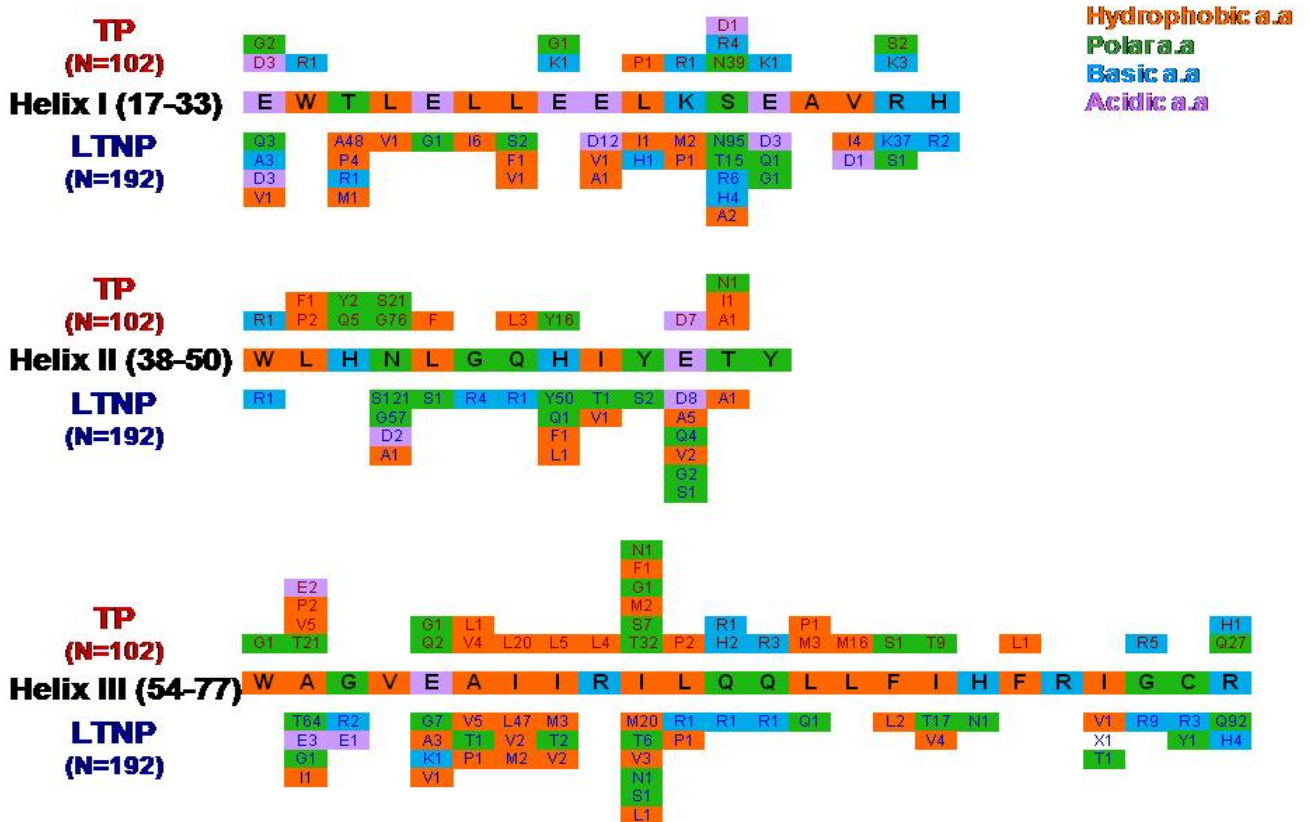
Figure 25. Comparison of aminoacid residues in helical domains of Vpr alleles derived from RP and LTNP.

Sequences of Vpr alleles across three helices of HIV-1 Vpr derived from rapid progressor (N=102) and long term non progressors (N=193) was compared based on polarity.

5.3.3 Identification of Vpr residues involved in Vpr oligomerization by BiFC

Representative Vpr mutants shown in Fig. 26A were selected for further biological evaluation. Vpr mutant molecules were cloned in venus-C and venus-N construct and verified for expression in HEK293T cells by transfection followed by immunoblot (Fig. 26B). Results indicate that all Vpr mutant chimeric molecules express the appropriate size protein. However, the expression

level of Vpr mutant molecules differ slightly compared to wild type. For instance, mutants A30L, Δ44, E48A and H71R showed 40% reduction and L68E exhibited 60% reduction compared to wild type Vpr, whereas MA remained the same as wild type. Control vector did not show any signal suggesting that Venus-chimeric Vpr mutant molecules are not defective in expression and/or stability.

(A)

```
NL4-3  MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS*
A30L   -----------------------------------L-------------------------------------------------------------*
Δ44    ---------------------------------------------------Δ---------------------------------------------*
E48A   ----------------------------------------------A-------------------------------------------------*
L68E   ----------------------------------------------------------------E-------------------------------*
H71R   -------------------------------------------------------------------R-----------------------------*
MA     -------------F----I-------N----R--E--G------------------------------------Q-----I---T---------*
```

(B)

(C)

	DAPI	BiFC	Vpr	Overlay
Vector				
Vpr^wt				
Vpr^A30L				
Vpr^Δ44				
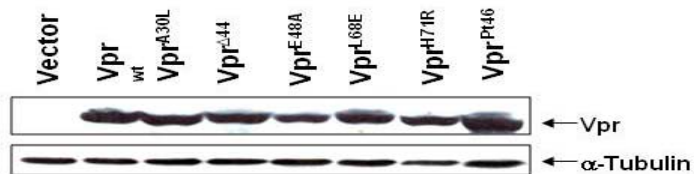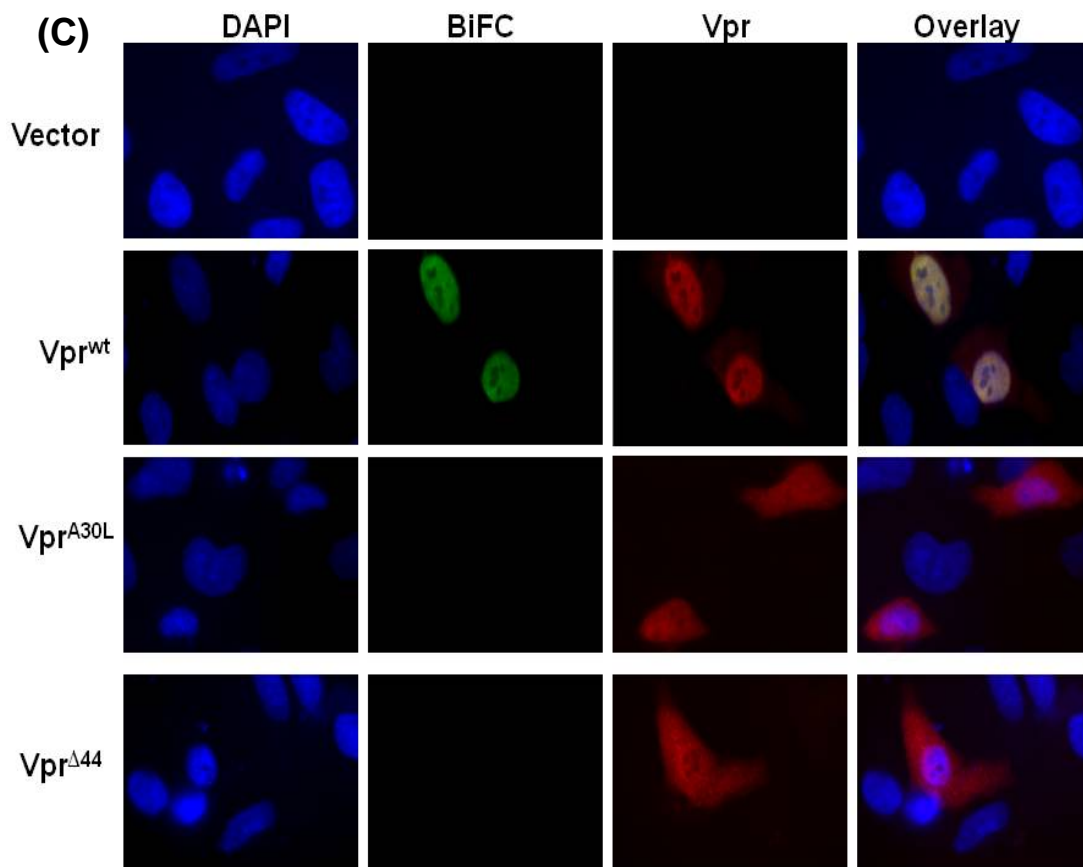
(Continued below)
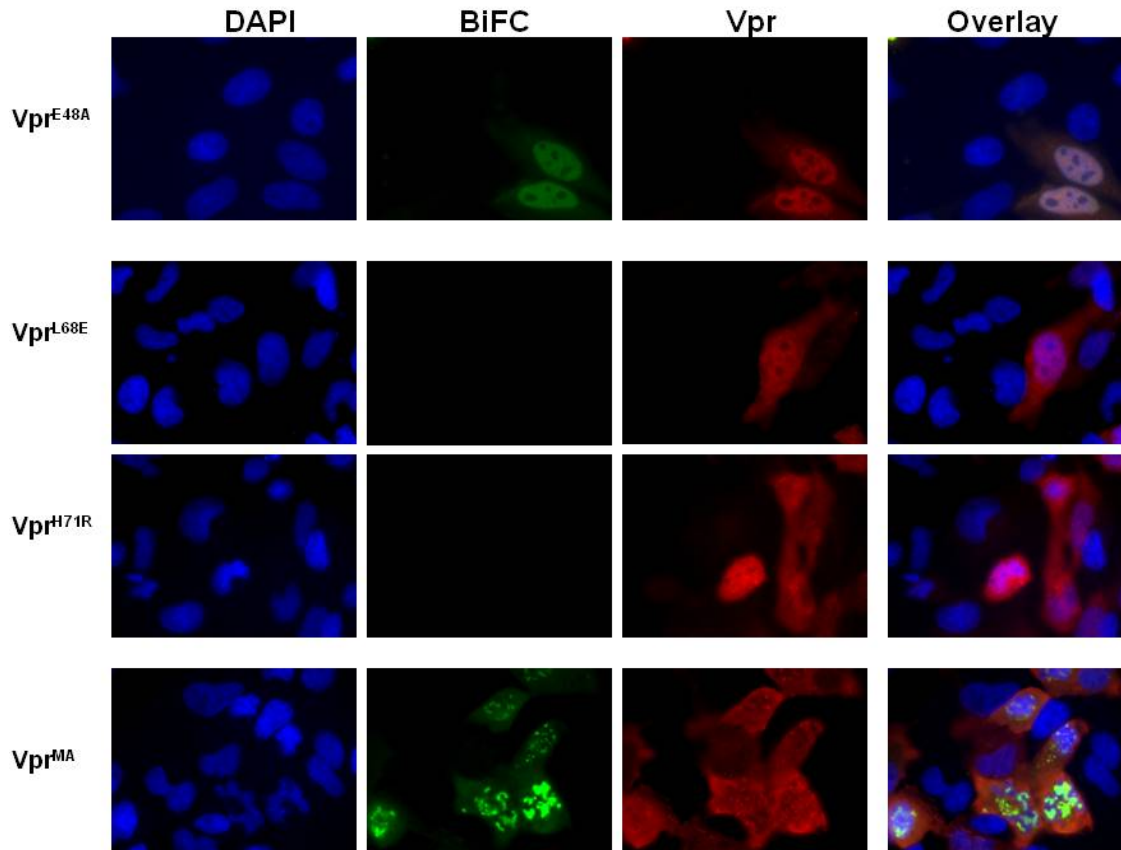
(D)
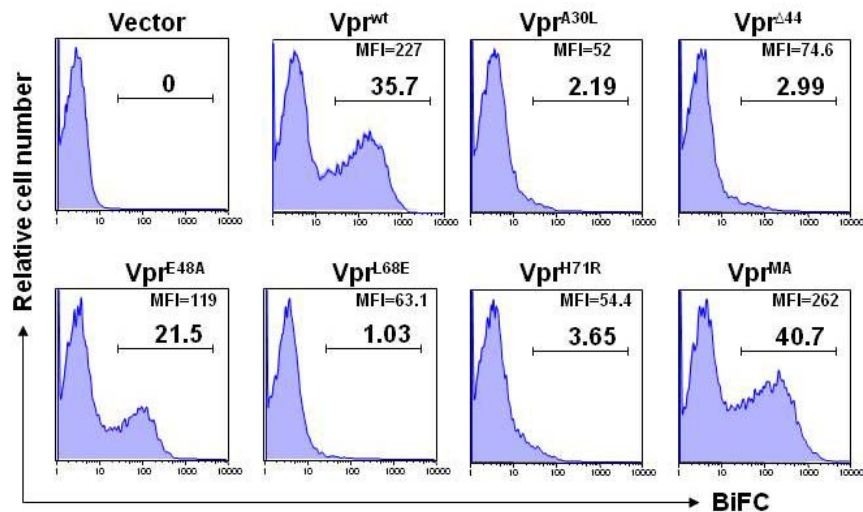
Figure 26. Identification of residues required for Vpr dimerization.

(*A*) *Schematic figure depicting Vpr mutants selected for further analysis. Substitution residue(s) are marked at appropriate place and a Δ represent deletion of a residue. MA, represent Vpr clone with multiple aminoacid (MA) changes in helix I and III. NL43 sequence was used as wild type clone. (**B**) Expression of Vpr mutants was assessed in HEK293T cells by transient transfection. HEK293T cells were transfected with Vpr mutant expression plasmids*

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*or vector control plasmid and assessed for expression by western blot using HA to detect fusion protein. Tubulin was used as a loading control. **(C)** Visualization of dimerization and subcellular distribution of Vpr mutant molecules was assessed by BiFC. HeLa cells were transfected with VC and VN combinations of Vpr mutants as described. Thirty-six hours posttransfection cells were stained for Vpr (shown in Red) and DAPI (blue) and imaged at 60X magnification. **(D)** Quantitation of dimerization of Vpr mutant molecules in HeLa cells. Cells were transfected with combination of VC and VN plasmids as described. Thirty-six hours post-transfection cells were assessed for BiFC by flow cytometry. Percentage of cells positive for BiFC and MFI are marked. Figure represents one of five independent experiments (n=5) with similar results.*

Next, we assessed the ability of Vpr mutants to form dimers/oligomers by BiFC analysis. The expression of Vpr molecules was also assessed from the same batch of cells by indirect immunofluorescence using HA antibody (to detect HA tagged Vpr) and compared with BiFC by microscopy (Fig. 26C). Results indicate that Vpr^{wt}, E48A and MA exhibited detectable BiFC signal, whereas mutants A30L, Δ44, L68E and H71R did not show BiFC positive cells, suggesting that these mutants are defective in dimerization. Importantly, staining for Vpr (panel Vpr in Fig. 26C) further confirmed the expression of Vpr protein suggesting that lack of dimerization is not due to lack of Vpr expression. Next, we assessed whether subcellular distribution of Vpr mutant molecules has any role in dimer/oligomers formation. BiFC positive Vpr molecules, Vpr^{wt}, and E48A show uniform nuclear distribution.. However, BiFC negative mutants, A30L, L68E and H71R also showed nuclear distribution similar to Vpr^{wt}. Role of specific residues of Vpr in dimerization/oligomerization was further confirmed by measuring the percentage and mean fluorescence intensity (MFI) of BiFC signal using flow cytometry (Fig. 26D). Results indicate that BiFC signal is detected in HIV-1 Vpr^{wt}, mutants E48A and MA, whereas A30L, Δ44, L68E and H71R did not show any significant signal. The low level of BiFC signal (<4%) observed in A30L, Δ44, L68E and H71R could be due to background or auto fluorescence. The percentage of positive cells and MFI is comparable in Vpr^{wt} and MA is 35.7% (MFI 227) and 40.7% (MFI-262), respectively, whereas 21.5% (MFI-119) of cells were BIFC positive in E48A.

5.3.4 Relevance of oligomerization in Vpr-Gag interaction and virion incorporation of Vpr.

Vpr interacts with HIV-1 Gag specifically through the p6 domain and packages into the virus particles in significant quantities [156, 162, 163]. Therefore, we assessed whether Vpr-Gag interaction is detectable in BiFC based live cell assay using Venus-Gag and Venus-Vpr plasmids. Combination of Venus-C and Venus-N plasmids expressing either Gag or Vpr was cotransfected and evaluated for BiFC signal by fluorescence microscopy. Subcellular distribution of Gag-Gag interaction and Gag-Vpr interaction resulted in cytoplasmic membrane localization, whereas Vpr-Vpr interaction resulted in nuclear localization (Fig.27A). Together, the fluorescence microscopic analyses reveal that Vpr interacts with Gag very specifically at the cytoplasmic membrane and this interaction results in differential localization of Vpr corresponding to virion incorporation and virus assembly.

(A)

VN-Gag+VC-Gag   VC-Gag+VN-Vpr^wt   VN-Vpr^wt+VC-Vpr^wt   HIV-1ΔR+VN-Vpr^wt+VC-Vpr^wt

(Continued below)

99

(B)
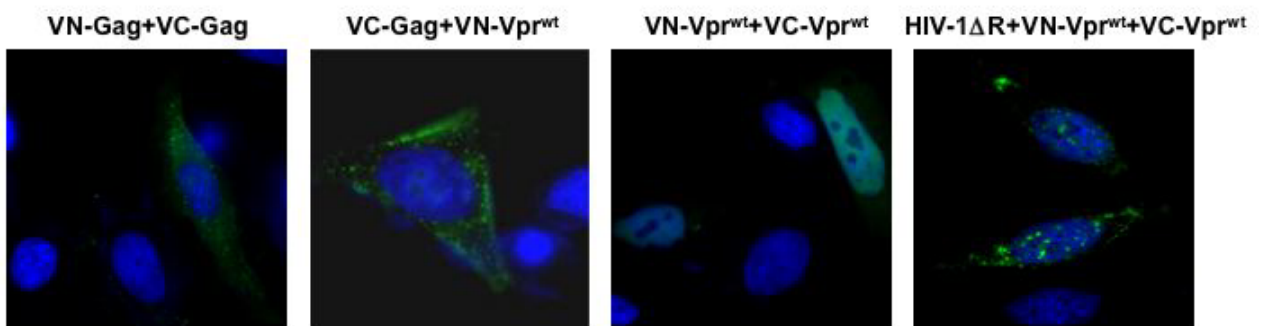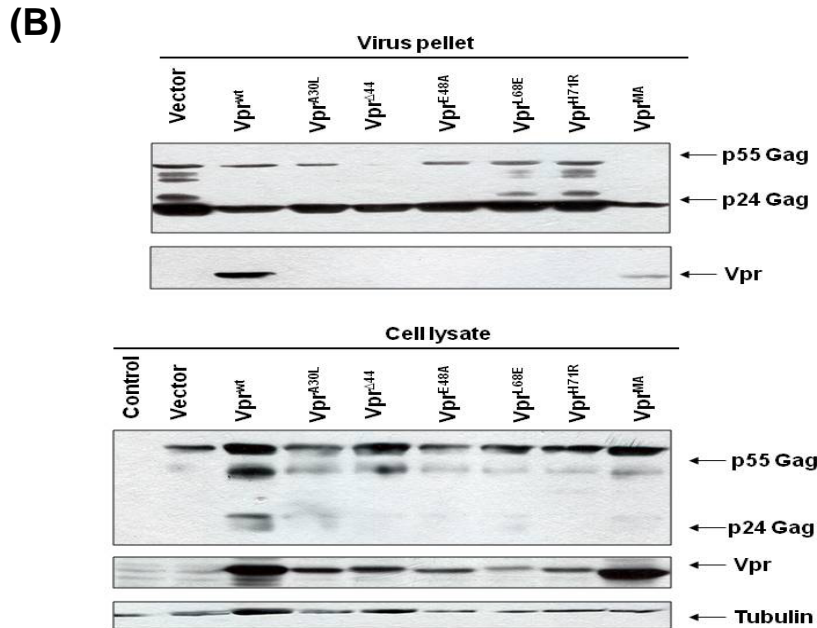
Figure 27. Role of HIV-1 Vpr oligomerization in incorporation of Vpr into virus particles :

Interaction of HIV-1 Gag and Vpr.

(A) Visualization of Vpr^{wt} and Gag interaction in HeLa cells by BiFC. HeLa cells were transfected with VN-Vpr^{wt} and VC-Gag expression plasmids. Thirty-six hours post transfection, cells were fixed, stained with DAPI and analyzed for presence and pattern of BiFC signal at 60X magnification. (B) Incorporation of Vpr mutant molecules in virus particle. Vpr plasmids were cotransfected with pNL43ΔVpr proviral plasmid in HEK293T cells. Forty-eight hours post-transfection, supernatant and cell pellet were lysed, subjected to SDS-PAGE electrophoresis and evaluated for presence viral proteins Gag and Vpr by western blot. Tubulin was assessed in cell lysate as a loading control.

Next, we assessed the ability of Vpr mutants to incorporate into virus particles. HEK293T cells were cotransfected with pNL43Δvpr proviral plasmid and Vpr expression plasmids or vector control. The amount of Vpr in cells and virus particles was quantitated by loading equal amount of total protein of the cell lysate or normalized using Gag-p24 antigen, respectively (Fig. 27B). Results indicate that expression of Vpr^{wt}, A30L, Δ44, E48A, H71R and MA is comparable in cell lysate (except L68E) suggesting that the expression and stability of these mutants are not altered entirely. Analysis of Vpr in virus particles revealed that Vpr^{wt} and MA incorporated into the virus particle, whereas Vpr mutants A30L, Δ44, E48A, L68E and

H71R did not incorporate into virus particles suggesting a role for these residues in Vpr-Gag interaction and virion incorporation. Together theses studies indicate that dimerization/oligomerization defective mutants are defective in virion incorporation suggesting that oligomerization is an essential feature for virion associated Vpr functions.

Though both Vpr^{wt} and MA incorporate in virus particles, it is important to note that incorporation of MA is significantly less compared to Vpr^{wt} although both express equally high level of protein. Further we noticed a consistent reduction in the amount of Gag released in the supernatant in presence of Vpr^{MA} clone, even when the Gag in the cell lysate was comparable with other mutants. Cell lysate from the same culture revealed accumulation of Vpr in the cells, suggesting that Vpr mutant MA might interfere with either Vpr-Gag interaction or virus release. To test these possibilities, we cotransfected VN-Gag and VC-Vpr^{MA} or VC-Gag and VN-Vpr^{MA} and assessed the distribution of BiFC and compared with the Vpr^{wt} counter part (Fig. 28A). Results indicate that distribution of Gag-Vpr^{wt} interaction resulted in cytoplasmic membrane distribution (top panels), whereas, Gag-Vpr^{MA} showed a condensed nuclear accumulation (Fig. 28A; bottom panels). To precisely quantitate this and to further delineate the role of this mutant in virus release and production, we transfected HEK293T cells with pNL43Δvpr-EGFP and pVpr^{wt} or pNL43ΔVpr-EGFP and pVpr^{MA} and measured the amount of p24 released in the culture supernatant and the amount present in the cell lysate by ELISA (Fig. 28B). Considering the total amount of p24 present in both supernatant and cell lysate as 100%, we assessed the fraction of p24 present in supernatant and cell lysate for each experiment. Results in Figure 28B, shows the average of p24 value seen in multiple experiments (N=6). Results indicate that compared to Vpr^{wt}, Vpr^{MA} showed 70-30% reduction in p24 release in the supernatant, whereas amount of p24 present in cell lysates showed a 3-4 fold increase. Together these studies suggest

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that hyper oligomeric property of Vpr^{MA} might sequester Gag within the cell and prevent the

release of virus particles.

(A)

(B)

Figure 28. Hyperoligomeric Vpr mutant induces sequestration of Gag.

(A) Interaction and subcellular distribution of Gag-Vpr^{wt} and Gag-Vpr^{MA} was assessed by BiFC. HeLa cells were transfected with combinations of Gag and Vpr^{wt} or Gag and Vpr^{MA} as described. Thirty-six hours post-transfection cells were fixed, stained with DAPI (blue) and imaged at 60X magnification. (B) Effect of hyper oligomeric Vpr mutant MA on virus/Gag release by ELISA. HEK293T cells were cotransfected with NL43ΔVpr-

that hyper oligomeric property of Vpr^{MA} might sequester Gag within the cell and prevent the

release of virus particles.

(A)

Figure panels labeled:
VN-Vpr^{wt} + VC-Gag (DAPI, BiFC, Overlay)
VC-Vpr^{wt} + VN-Gag (DAPI, BiFC, Overlay)
VN-Vpr^{MA} + VC-Gag (DAPI, BiFC, Overlay)
VC-Vpr^{MA} + VN-Gag (DAPI, BiFC, Overlay)

(B)

$* \, p < 0.05$

Gag in supernatant
Gag in cell lysate
(bar chart: WT, MA)

Figure 28. Hyperoligomeric Vpr mutant induces sequestration of Gag.

(A) Interaction and subcellular distribution of Gag-Vpr^{wt} and Gag-Vpr^{MA} was assessed by BiFC. HeLa cells were transfected with combinations of Gag and Vpr^{wt} or Gag and Vpr^{MA} as described. Thirty-six hours post-transfection cells were fixed, stained with DAPI (blue) and imaged at 60X magnification. (B) Effect of hyper oligomeric Vpr mutant MA on virus/Gag release by ELISA. HEK293T cells were cotransfected with NL43ΔVpr-

5.3.5 Role of oligomerization in Vpr-mediated cellular functions.

Vpr is known to induce cell cycle arrest and apoptosis in both infected and exposed target cells as well as modulate several viral and host transcription factors [3, 74, 164, 165]. To understand whether defective or altered oligomerization affect the well-established Vpr-mediated functions, we evaluated the role of the Vpr mutants on apoptosis (Fig. 29A). Our analyses indicate that Vpr^{wt} at a concentration of 2.5µg induced apoptosis in 71% of the cells, whereas the vector transfected cells show 8% (10-fold low). Among the mutants, MA showed similar level (61%) to wild type Vpr and Δ44, E48A, L68E and H71Y induced 40%. Importantly A30L is less apoptotic (19%) compared to all the tested mutants. These studies suggest that Vpr oligomerization might enhance apoptosis, however it is not absolutely essential for Vpr-induced apoptosis.

HIV-1 Vpr has been established as a transcriptional regulator as well as a coactivator molecule [32, 150]. Vpr activates both viral and cellular promoters and regulate gene expression with in the context of infection as well as expression. Next, we assessed the significance of oligomerization defective mutants on Vpr induced HIV-1 LTR mediated transactivation (Fig. 29B). Results indicate that oligomerization positive wild type and E48A showed 4-fold increase in LTR mediated activity, whereas MA did not show LTR transactivation (<1 fold). Oligomerization defective mutants did not show any significant difference compared to wild type, MA or E48A.

Similarly, we also evaluated the ability of Vpr oligomerization on cellular promoters using NF-κB-luciferase reporter as described in methods. Vpr is known to suppress the NF-κB mediated transactivation [31, 109]. Ability of Vpr mutants to inhibit NF-κB mutants were assessed in HeLa cells and the results indicate that Vpr mutants (positive and negative for dimerization/oligomerization) did not show any significant difference (Fig. 29C). Vpr wild type, Δ44, E48A and H71Y showed 60% reduction compared to vector cotransfected culture, L68E did not show any inhibition. However, we observed that the hyperoligomeric mutant MA and oligomerization defective A30L inhibited 30-40% Vpr-mediated HIV-1 LTR transactivation and reversed Vpr-induced NF-κB suppression. Collectively, these results suggest that oligomerization might have an impact on cellular functions and transcriptional regulation, however it is not absolutely necessary for its function.
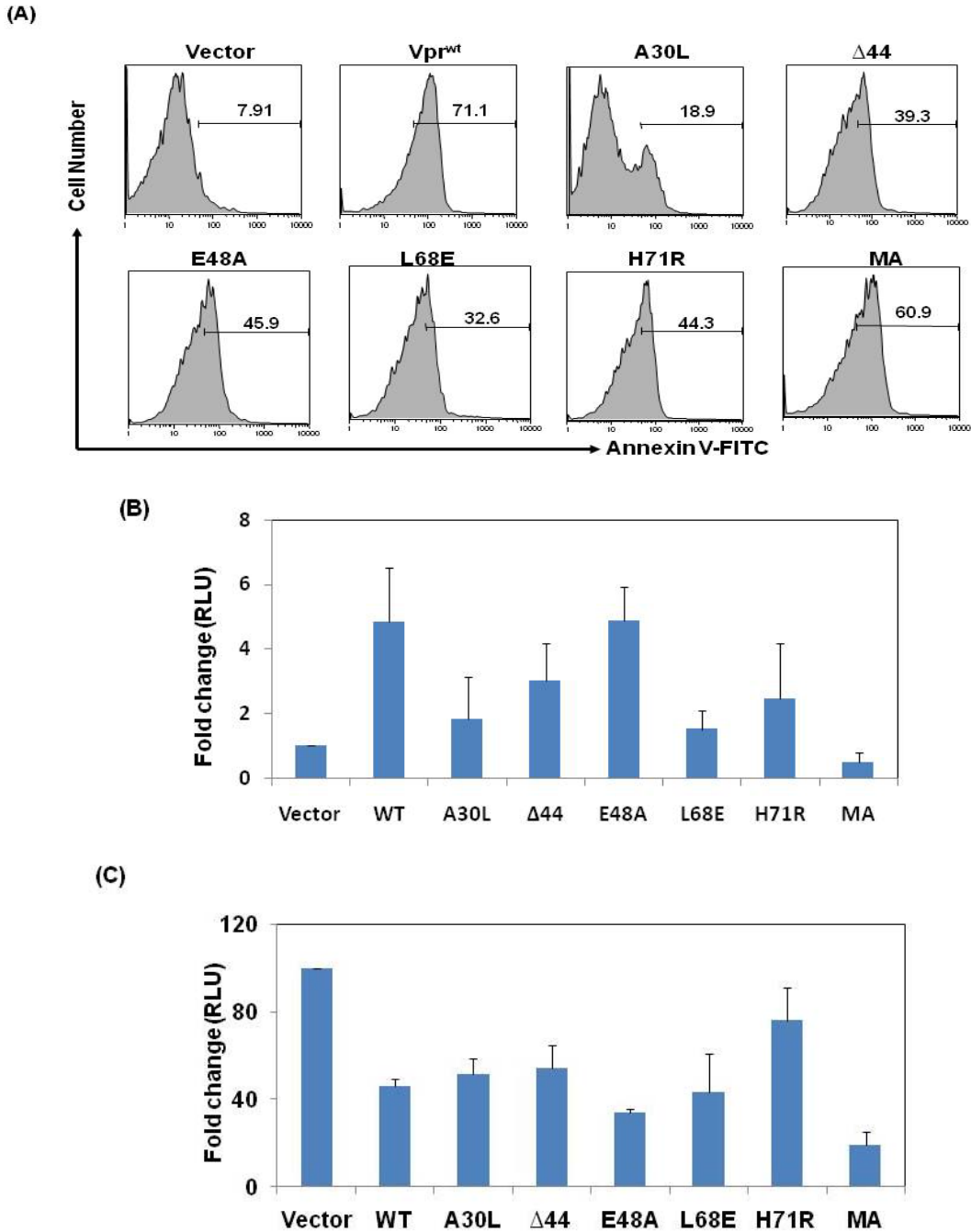
Figure 29. Role of Vpr Oligomerization in Vpr mediated cellular functions.

*(A) Evaluation of Vpr mutant molecules to induce apoptosis in HeLa cells. Cells were transfected with Vpr^wt, Vpr mutants plasmids or vector control plasmid, forty-eight hours post transfection the cells were assessed for apoptosis by flow cytometry following Annexin V staining. (**B &C**) Evaluation of oligomerization defective Vpr mutants in viral and host cellular gene transcription. Hela cells were transfected with Vpr mutant expression plasmids and (B) HIV-1 LTR-luciferase reporter plasmid or (C) NF-kB-luciferase reporter plasmid, forty eight hours post-transfection, the assay was performed as described in Materials and Methods.*

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5.4   DISCUSSION

HIV-1 and 2 are members of lentivirus family of retroviruses and are grouped as complex retroviruses. The unique feature of this group in comparison to simple retroviruses is that the viral genome codes for several proteins in addition to the core structural proteins. In this regard, HIV-1 is known to code for six auxiliary proteins (Vif, Vpr, Tat, Rev, Vpu and Nef) besides the structural proteins. Previous studies have demonstrated that auxiliary proteins play an essential role in HIV-1 replication and pathogenesis. Our laboratory has been interested for several years in evaluating the contribution of auxiliary proteins including Vpr. In this study, we have analyzed the requirement of dimerization/oligomerization property of Vpr and its relevance to the functions of Vpr. Specifically Vpr shares this feature with other auxiliary proteins such as Vif, Rev, Vpu, and Nef.

HIV-1 Vpr is a small oligomeric protein that plays an important role in HIV pathogenesis [20, 70, 145, 151, 166]. The underlying reasons for selection of Vpr as a target for the present studies are the following: (i) Vpr is a virion associated protein; (ii) Vpr plays a critical role for the replication of virus in macrophages and positively regulates viral replication in T cells; (iii) Vpr is a transcriptional activator of HIV-1 and heterologous cellular genes; (iv) Vpr inhibits proliferation of cells at G2/M phase; (v) Vpr induces apoptosis in diverse cell types including T cells and neurons; (vi) Vpr exhibits immune suppressive effects. Further, studies from non human primates and analysis of viral genes in long term non progressors suggest a good association between defective Vpr and delayed progression of the disease [70, 167, 168]. More importantly several Vpr-mediated functions are induced by both cell-associated and virion-associated Vpr [3, 20, 169]. Together these studies point out the biological significance of virion associated factors and its role in early infection. Therefore, understanding the role of

oligomerization in Vpr functions and disease progression will provide a novel target for the development of therapeutics in the future.

The dimerization/oligomerization feature of Vpr was evaluated by using a complementation system based on Venus reporter. A strategy involving the generation of chimeric Venus-Vpr protein has allowed us to monitor dimerization/oligomerization in live cells. This system has the sensitivity to detect Vpr-Vpr and Vpr-Gag interactions. While dimerization/oligomerization of Vpr has been documented previously, the domains/residues in Vpr required for this feature are not clear. To address this, we have utilized the conservative nature of specific residues in Vpr by analysis of the patient derived Vpr sequences representing all clades (n=1223). Several mutant Vpr molecules were generated containing alterations in the selected residues. The results regarding the expression and steady state level of Vpr indicated that mutants lacking the ability to oligomerize exhibit a pattern similar to that of wild type Vpr. This observation suggests that monomeric Vpr molecules are stable in cells.

HIV-1 Vpr is one of the non-structural proteins that is packaged in significant quantities in virus particle. Virion-associated Vpr is present in the infected cells prior to *de novo* synthesis and is known to cause the host cellular dysfunctions during early infection [20, 145, 166]. Studies have indicated that the p6 domain of Gag is critical for the incorporation of Vpr into virus particles[8, 68, 156]. Published studies using Vpr molecules with mutations in alpha helices indicate that helices I, II and III may be involved in Vpr oligomerization function, however, whether Vpr oligomerization is a prerequisite for virion incorporation is unknown. As expected, chimeric Venus containing wild type Vpr and chimeric Venus containing Gag resulted in the reconstitution of Venus with fluorescence suggesting an interaction between these two proteins. On the other hand, chimeric Venus containing mutant Vpr failed interact with Gag.

Vpr mutants which showed dimerization/ oligomerization negative phenotype also failed to incorporate into virus particles. Several studies have reported that virus particle contains around 250 molecules of Vpr in comparison to approximately 2500 molecules of Gag protein. This suggests that low amount of Vpr to Gag may be due to the interaction restricted to the specific configuration of Gag. Oligomerization defective mutants, A30L, Δ44, L68E and H71R lack the ability to interact with Gag and incorporate into the virus particles. This defect has significant relevance with their ability to activate HIV-1 LTR promoter, suggesting that Vpr oligomerization might be directly linked to pathogenesis and disease progression.

HIV-1 Vpr is known to modulate several host cellular functions such as cell cycle arrest, apoptosis and transcriptional regulation of cellular genes in the presence and absence of other HIV-1 proteins [74, 109, 164]. Many of these functions are induced through their interaction with cellular partners and are mediated through different domains/residues of Vpr [170]. However our in depth analyses on Vpr-dimerization and cellular functions (apoptosis and transcriptional activation of cellular promoters) suggest oligomerization positive Vpr (Wild type and MA) showed an enhanced apoptosis indicating a correlation between oligomerization and Vpr mediated cellular functions.

An understanding of HIV-1 Vpr functions and its properties, in our view, is likely to shed light on the mechanisms involved in Vpr incorporation into the virus particle and how oligomerization feature influences virus replication and other Vpr mediated functions. These studies may further provide a target for the development of potential therapeutic agents including small molecules against Vpr-Vpr interaction, Vpr-Gag interaction, virion incorporation and virus replication in primary macrophages and T cells.

6.0 OVERALL DISCUSSION AND FUTURE DIRECTION

6.1 SUMMARY OF FINDINGS

CD4+T cells have a central role in induction and homeostasis of the immune response, and are also the major target cells for HIV. HIV has devised mechanisms to subvert the immune system to further its cause of survival and dissemination, by utilizing the CD4+ T cells. HIV-1 Vpr is one of the accessory genes which is essential for the virus survival *in vivo* and has is incorporated in virus particles in adequate amount, suggesting a role for this protein in the early phase of infection. Being a soluble protein with an ability to transduce across cell membrane, Vpr can potentially affect uninfected/non target bystander cells. We hypothesize that HIV-1 Vpr alters the functions of both infected and bystander T lymphocytes, utilizing direct and indirect mechanisms, and these Vpr-mediated effects contribute inpart for the immune dysregulation and aid in viral dissemination. The specific aims of this proposal are: (i) Assess the immune modulatory effects of HIV-1 Vpr in infected and bystander T-lymphocytes *in vitro*; (ii) Understand the role of HIV-1 Vpr in T lymphocytes, Natural Killer (NK) cells and Dendritic cells (DC) interactions; (iii) Analyze the Structure-Function role of HIV-1 Vpr in immunopathogenesis, virus replication and disease progression, we mainly studied the role of Vpr oligomerization in its functions. To address these aims, we utilize HIV-1wt and HIV-1ΔVpr viruses and compare the difference in the effects of these viruses under physiologically relevant

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in vitro conditions. The differences observed in the effect of these two viruses can be attributed to Vpr provided that the infections in both the experimental groups are similar. In some studies, to distinguish the productively infected cells, we employed EGFP reporter viruses. The reporter virus helped us to understand the effects of Vpr in infected and bystander cells by using flow cytometry, immunofluorescence microscopy and also aided in sorting of productively infected cells by FACS sorting for further DNA and RNA analysis. Results indicate that HIV-1 Vpr can differentially regulate the surface expression of T cell costimulatory molecules, CD28 and CTLA-4 in the infected cells. There is downregulation of stimulatory costimulatory molecule, CD28 and upregulation of inhibitory molecule, CTLA-4 on infected cells. Also Vpr inhibits IFN-γ production in infected T cells, this can be explained by the ability of Vpr to disrupt cell signaling in T cells at multiple levels. Vpr modulate the expression of cell surface molecules, which are critical for transduction of signal following interaction with their counterparts. , Downstream in the signaling pathway, Vpr inhibits nuclear translocation of p56 portion of NF-κB. By augmenting the production of TGF-β and inducing chronic expression of NK activating receptor ligands, MIC A/B, hULBP-1 and hULBP-2 on the bystander virus/ Vpr protein exposed cells, without significantly altering the MHC class I A,B,C molecules, Vpr inhibits NK cell function. Results suggest that HIV-1 exposure of CD4+ T cells is sufficient for these cells to inhibit NK cell function. The loss of NK cells to lyse specific target cells was associated with reduced surface expression of degranulation marker, CD107a and reduced IFN-γ production. Preliminary results suggest the role of heat shock elements in this Vpr mediated upregulation of NK cell ligands. Previous studies from our laboratory and others have shown that Vpr directly impairs DC cell maturation and alters the cytokine secretion profile. Known that bi-directional signaling during T cell-DC interaction is critical for induction of immune response, and since

Vpr was able to differentially modulate surface expression of CD28 and CTLA-4, we evaluated the role of Vpr to indirectly modulate DC function through T cells. HIV is reported to convert immunological synapse to virological synapse, thus increasing the efficiency of virus dissemination. The ability of virus to exploit these mechanisms has been described as the virus employing the DC as "Trojan Horse" to protect itself from the host defense mechanisms during transit. Even though we were not able to detect a role for HIV-1 Vpr in immunological interaction of T cell-DC, we found that the interaction was exploited by the virus to disseminate. Previous report suggest that HIV virus infects DC in trans and stays protected with in the intracytoplasmic compartment of DC till it comes in contact with a T cell. Interaction of DC with the T cell, activates the antigen specific T cells and also infects the T cell. We report that HIV-1 virus can transfer from infected T cell to DC during T cell-DC interaction and this process is independent of viral envelope or known receptors for HIV, including DC-SIGN, CD4, CXCR4 and CCR5. Results from cycloheximide studies clearly suggest that EGFP+ DC is not due to antigen uptake or due to cells sitting together or cell fusion, but due to denovo synthesis of EGFP in DC. Further the cis infection in DC was confirmed by detecting integrated proviral DNA in sorted EGFP+ DC. Based on the direct correlation between the DC infection and ability to acquire cell associated antigen, we propose a model where HIV is transferred to DC independent of virus envelope, when DC interacts with an infected cell, and acquires cell associated antigens as part of their role in surveillance mechanism. More studies have to be undertaken to identify the molecules involved in these antigen uptake processes. Finally to understand the role of Vpr structure and function we compared Vpr alleles obtained from well-defined Long term non-progressors (LTNP) and rapid progressors (RP). Even though there were variation in the Vpr sequence at nucleotide level, there was a greater degree of conservation at the structural level

where amino acids were conserved based on their hydrophobicity index and tend to remain close to related aminoacids. Vpr alleles from LTNP were less conserved structurally than the alleles from RP, suggesting a role for Vpr in long term non progression. Vpr is known to form oligomers in vitro and in vivo. Results from Live cell based Bimolecular Fluorescence Complementation (BiFC) assay helped us to identify the critical residues involved in Vpr oligomerization. Results suggest that Vpr oligomerization is essential for Vpr incorporation into virus particles. Oligomerization is also critical in other vpr function, including apoptosis and regulation of transcription from host and viral promoters, but a direct correlation cannot be seen. Further we identified a Vpr allele, which has multiple mutations, was forming higher orders of oligomers and was detrimental to the cell. The aggregates formed due to the hyperoligomerization property of the Vpr allele was able to sequester the interacting HIV-1 Gag proteins and interfere with the release of Gag. Understand these critical roles of Vpr in viral pathogenesis, empower us to devise novel strategies to combat HIV infection.

6.2   PUBLIC HEALTH SIGNIFICANCE

HIV/AIDS pandemic is one of the major public health challenges of the present generation. With an estimated 30-36 million people infected with HIV/AIDS around the world, and no preventive vaccine available, there is urgency for novel therapies targeting HIV-1 infection. Greater fraction of this disease spans the developing world, this mandates need for therapeutics, which are both inexpensive as well as easily accessible. Though primary prevention by health education and awareness is possible, it is difficult to achieve and cannot be the only focus to curtail infection. While current anti-retroviral therapy has delay the progression of disease, effective suppression

or eradication of infection is not possible. Also, presently available anti retroviral therapies are associated with adverse effect and the patient compliance of treatment is low. This obliges a better understanding of viral pathogenesis to develop novel therapeutics, which can aid in primary prevention and spread of infection across the globe. HIV-1 Vpr is one of the accessory genes which is essential for viral survival in vivo. Vpr is known to have diverse effect during HIV pathogenesis, including apoptosis, suppression of immune system and aiding the virus at multiple steps of infection. Understanding the mechanistic of the Vpr pathogenesis will help us develop new class of anti-virals targeting this critical protein at different stages of viral life cycle. Such a therapy can supplement the available HAART regimen to make it more effective and more patient compliance.

6.3   FUTURE DIRECTIONS

Results from studies evaluating direct effect of Vpr on T cells during HIV-1 infection, suggest a role for HIV-1 Vpr in differential regulation of major T cell costimulatory molecules in infected T cells, it will be interesting to further understand the mechanism involved in this process. Results from limited number of donors suggest that Vpr regulates these critical signal transduction molecules at the transcriptional level. Identifying the mechanism(s) involved in Vpr-mediated immune regulation will improve our understanding of viral function, and assist in the development of immunotherapeutics and antiviral strategies for HIV-1-infected individuals. Further, evaluating whether Vpr is able to regulate these genes reciprocally and/or independently will help us to understand the regulatory mechanism(s) involved in the expression of these counter regulatory molecules. Developing agents which can specifically modulate expression of

certain immunological molecules will be valuable tools in dissecting the pathogenesis of immunological disorders and can be useful as therapeutics in repressing hyper reactive immune conditions and in transplantation biology.

Even though we were unable to identify a role for Vpr mediated differential regulation of costimulatory molecules in T cell – DC interaction, studies evaluating the effect on tryptophan metabolism and effects on cytokine secretion profile in DC may be useful.

We have shown that Vpr has a role in inhibition of IFN-γ and upregulation of TGF-β in infected T cell cultures, effect on other critical cytokines and chemokines will enable us to understand the role of Vpr in HIV-1 immunopathogenesis in a better way.

NK cell study results suggest a role for HIV-1 Vpr in inhibition of NK cell function, but interestingly even Virus Like Particles lacking Vpr, do have a role in upregulation of NK cell activating receptor ligands and can inhibit NK cell function, in long time cultures, suggesting a role for viral proteins, Gag and Envelope. Alternatively there can be other cellular factors involved in NK cell dysregulation. It is very tempting to predict and evaluate the role of heat shock proteins in triggering of NK cell receptor ligands. Heat shock proteins are reported to induce NK cell activating receptor in exposed cells, and analysis of MIC A/B promoter confirms the presence of heat shock elements in the promoter of the genes. Also reports suggest that Heat shock proteins can interact with Gag and incorporate into virus particles. Understanding the factors involved in HIV-1 mediated inhibition of NK cell functions and identifying the cellular players involved will help us develop novel immunotherapeutics favoring cells of the innate immune response,

Studies involving infected T cell – DC shows that virus is transferred from T cells to DC. Though we have proposed a model where the virus is transferred from interacting T cell to DC,

more detailed studies are necessary to clearly delineate the pathways involved in the dissemination of virus from T cell to DC. Another interesting observation in these experiments was that, a good number of DC uptake antigen from the interacting cell, relatively fewer numbers of DC are productivey infected, suggesting the presence of restriction factors in DC. Identifying the restriction factors will be critical for these expeiments. Our model also predicts a role for single and double circle LTR in transmission of vius from T cell to DC and additional experiments are required to ascertain the role of LTR circles and associated resistance to current anti-retroviral therapeutics.

This dissertation mainly focuses on role of Vpr and Vpr dysregulated T cell on other immune cells following their interaction with T cell. But more studies have to be conducted to understand the role of HIV-1 Vpr in the NK cell-DC cross talk and viceversa. Such a study will help us to identify the cellular factors involved during the cross talk essential to link the innate and adaptive immune responses to the invading organism.

Finally studies aimed at understanding the role of structure-function of Vpr suggest that oligomeriation of Vpr is essential for multiple activities of HIV-1 Vpr. Targeting the oligomerization property of HIV-1 Vpr seems promising in development of therapeutics disrupting Vpr function. The BiFC assay used in these experiments can be easily adapted to screen huge compound libraries in a high throughput format. Similar assays can be developed for other viral proteins, which are known to interact with other partners or with themselves to form oligomers. Results from Vpr allele MA provide insight in to the effects of viral proteins which form higher order of oligomers. Vpr-MA allele can sequester the interacting gag protein in the aggregates. As an extension of this phenomenon observed in Vpr mutant MA, therapeutics can be developed which either crosslinking or cause misfolding of viral proteins and induce them to

form aggregates. Thus these compounds not only affect the normal function of the target viral protein but also interfere with the normal functioning of other proteins which are known to interact with the target proteins., Such an approach will be detrimental to the virus infected cells. Specificity for the compounds to affect the target viral protein with minimal and/or no toxic effect on cells can be evaluated by Live Cell Based High Throughput Assay.

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