DELINEATING THE ROLE OF SIV-VPR AND VPX ON DENDRITIC CELLS, NK CELLS, AND IMMUNE FUNCTION

by

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DELINEATING THE ROLE OF SIV-VPR AND VPX ON DENDRITIC CELLS, NK CELLS, AND IMMUNE FUNCTION

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Studies of viral accessory genes have progressed in order to understand pathogenesis and develop effective therapeutics and vaccines. For human immunodeficiency virus type-1 (HIV-1), one such gene receiving special focus is *vpr*. Vpr has been implicated in dysregulation of host cellular events (including cell cycle arrest and apoptosis), infection of non-dividing cells, and increased viral replication in infected T cells. In simian immunodeficiency virus (SIV), a similar gene is seen in a slightly different form, including *vpr* as well as a duplicate, *vpx*. In SIV, these two genes have been shown to split the functions of HIV-1 *vpr*. In order to use SIV as a model for HIV-1, it must be determined which SIV gene is responsible for mediation of different functional effects.

HIV-1 *vpr* has been shown to downmodulate surface markers on dendritic cells and alter cytokine environments *in vivo*. Studies have shown that HIV-1 *vpr* pushes natural killer cells into anergy, rendering them non-functional. Results presented in this study indicate SIV infection also results in these effects, but responsibility for these effects is split between SIV *vpr* and *vpx*. The *vpx* gene appears to play a role in downmodulation of surface receptors on dendritic cells and changes the cytokine environment within the dendritic cells. The *vpr* gene, however, appears to be responsible for decreased functionality of NK cells, leading to a non-functional

anergic state. These findings suggest SIV *vpx* and *vpr* cause similar effects compared to HIV-1 *vpr* and, as expected, the SIV genes split the functions of their HIV-1 homolog.

Statement of Public Health Relevance: HIV infection and disease is a growing epidemic and it has become increasingly apparent that *in vitro* studies are not sufficient to provide the data needed to create an effective vaccine. Because vaccine research cannot be performed on human subjects, the best mode for transition would be a shift to *in vivo* studies on non-human primates using SIV as a model for HIV-1 infection and disease. Before this can be adopted, it will be necessary to show HIV-1 and SIV have similar effects *in vitro* on immune cells and can be used interchangeably.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 HIV AND SIV: AN INTRODUCTION

1.1 HIV

The World Health Organization currently estimates that 42 million people worldwide are infected with the human immunodeficiency virus (HIV) and that there are 14,000 new infections each day [10]. The distribution of these cases, however, is disproportionate, with most infections occurring in Sub-Saharan Africa and South East Asia (Table 1).

Table 1. ***Regional HIV and AIDS Statistics (2007)***
There are currently an estimated 42 million HIV/AIDS cases around the world. The distribution of these cases is uneven and certain areas carry the bulk of the total cases around the world. Adapted from WHO statistics.

Region	Adults & Children Living with HIV/AIDS	Adults & Children Newly Infected	Adult Prevalence	Deaths of Adults & Children
Sub-Saharan Africa	22.5 million	1.7 million	5.0%	1.6 million
North Africa & Middle East	380,000	35,000	0.3%	25,000
South and South-East Asia	4 million	340,000	0.3%	270,000
East Asia	800,000	92,000	0.1%	32,000
Oceania	75,000	14,000	0.4%	1,200
Latin America	1.6 million	100,000	0.5%	58,000
Caribbean	230,000	17,000	1.0%	11,000
Eastern Europe & Central Asia	1.6 million	150,000	0.9%	55,000
Western and Central Europe	760,000	31,000	0.3%	12,000
North America	1.3 million	46,000	0.6%	21,000
Grand Total	33.2 million	2.5 million	0.8%	2.1 million

Individuals history of a sexually transmitted disease, men and women who have unprotected sex with multiple partners, men who have sex with men, injection drug users who share needles, and

health care workers with needle stick exposure are at the greatest risk for exposure to the virus [11]. Additionally, the virus can be passed horizontally from mother to child during childbirth as well as through breastfeeding [11]. There is currently no known cure or preventative vaccine for HIV and the only known effective prevention of HIV transmission is abstinence from sexual intercourse and the use of a condom [63]. Infected individuals can, however, now live longer and more normal lives with the use of highly active antiretroviral therapy (HAART). This drug regimen targets different parts of the viral lifecycle in an effort to prevent rapid T-cell loss by preventing reverse transcription, integration, or maturation of the virus particle [2]. Using HAART during pregnancy has also helped to nearly eliminate horizontal transmission from mother to child during childbirth within the United States and has helped to significantly decrease cases in the developing world [64]. Despite these advances, more work needs to be done to decrease the number of new infections each year.

HIV is a member of genus Lentivirus and the family *Retroviridae*. The virus is a 9.8 kilobase single stranded RNA genome, composed of nine genes coding for a different structural (*gag*, *env*, and *pol*), regulatory (*tat* and *rev*), and accessory (*vif*, *vpr*, *nef*, and *vpu*) proteins (Fig 1).

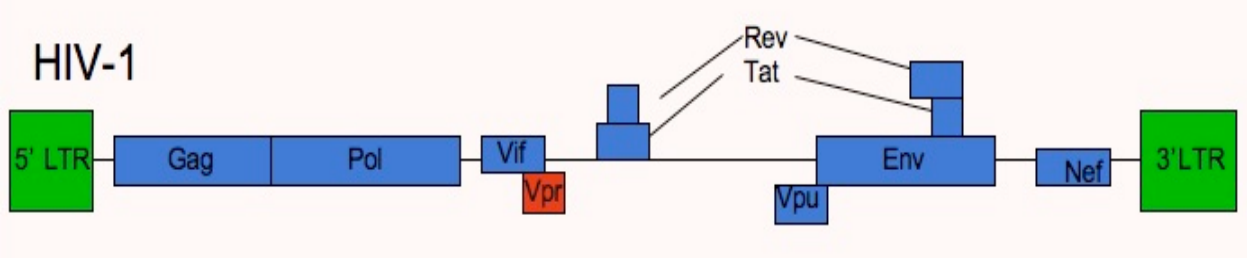


Figure 1. ***Genomic organization of HIV-1***
Schematic depicting the organization of the HIV-1 genome, including all structural and non-structural accessory proteins necessary for infection and replication of target cells.

Long terminal repeats (LTRs) flank both ends of the genome and serve as the promoter for viral transcription [2]. HIV-1 has an envelope, which is used to gain entry into a cell [2]. Specifically, the gp120 envelope protein uses the CD4 receptor CXCR4 and CCR5 co-receptors to enter a host cell. Upon entry, the HIV-1 genome is reverse transcribed into DNA in the cytoplasm of the cell [2]. Through the action of various accessory proteins, the viral DNA then enters the nucleus, where it can integrate into the host chromosomal DNA [2]. The virus targets a number of different cell types, including CD4+ T cell and macrophages, where post-infection latent reservoirs are established, causing both the initial asymptomatic period and an inability to fully rid a patient of the virus [2]. This reservoir effects a number of different cells, including dendritic cells (DCs) and natural killer (NK) cells, that play crucial roles in the immune response as well as the spread of the virus [2]. While only a small percentage of DCs become infected, many more are effected by the presence of virus, viral proteins and virally infected cells within the microenvironment [2]. These steps are depicted below by Nielsen *et al* (Fig. 2) [2].

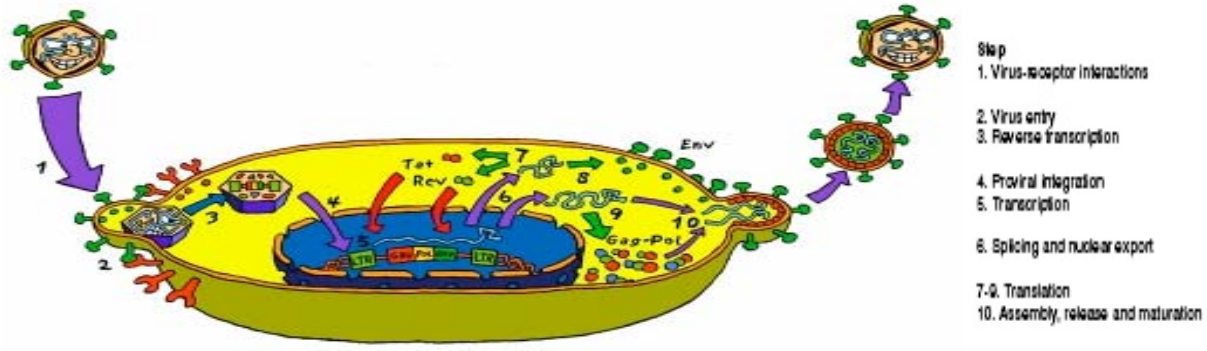


Figure 2. ***HIV Life cycle***
Lifecycle of HIV-1, used with permission. Virus enters via virus-specific receptors CD4, CCR5/CXCR4. The virus then uncoats within the cytoplasm and reverse transcription of the genome occurs. The genome is then moved to the nucleus with the aid of accessory proteins where it then integrates into the host cellular DNA. Host proteins transcribe the genome into RNA, which can then be used for translation of viral proteins and also packaged into viral particles for release. The virus assembles at the cell membrane where it then buds out and matures and can then go on to infect other cells.

HIV-1 accessory proteins play important roles in the development of pathogenesis and the virus' evasion of the immune system [1, 3]. Many of the accessory proteins are crucial for infection and deletion of these proteins results in a dead or non-infections virus [65].

Table 2. ***HIV-1 Protein Function*****.**
All HIV-1 proteins and their associated functions.

HIV-1 Protein	**Function**
Vif	Viral infectivity factor; RT complex stability
Nef	Negative Factor; Downregulates CD4 and MHC I expression; plays role in viral pathogenesis
Gag	Comprises the matrix, capsid, nucleocapsid structural proteins; p24 protein
Pol	Precursor to Protease, integrase, and reverse transcriptase
Rev	Regulator of expression; promotes nuclear import of viral RNA
Tat	Transcriptional activator; enhances gene transcription
Vpu	Down-modulates CD4 and enhances viral release
Env	Comprise the viral envelope and promote cellular attachment; gp-41 and gp-120;
Vpr	Viral protein R; plays role in transport of pre-integration complex, apoptosis and transactivation of cells, as well as G2 cell cycle arrest

HIV is known to infect CD4+ T-cells and kill them through viral replication [4]. This process directly leads to the illness known as acquired immune deficiency syndrome (AIDS) and other complications such as decreased ability to fight off infections. HIV can also infect other immune cells such as macrophages and DCs, which present antigens to CD4+ and CD8+ T-cells. This passage of virus leads to infection of CD4+ T-cells, which allows virus propagation. Dendritic cells, which typically are not infected, can also acquire live virus and present it to T-cells, infecting them in the process [7]. These disease pathways lead to the clinical symptoms seen in HIV-1 infected patients, such as increased susceptibility to opportunistic pathogens.

The infection process of HIV-1 has been studied extensively, and has lead to advances such as HAART that allow for better viral control. Despite these advances, though, clinical responses to

HIV infection are still limited to treatment confining prevention efforts to public health efforts such as education and condoms. While the development of a universal vaccine is still only a distant goal, because HIV-1 is only able to infect humans, an animal model is not available for the study of HIV-1 infection. The current in vitro models have allowed for progress but are limited in application, as they only distantly mimic what actually happens during infection, looking at events singly and not within the context of the rest of the body and immune system. This limitation poses the biggest obstacle in the study of HIV-1 pathogenesis and the development of an effective vaccine strategy. The best and most physiologically relevant model we have is simian immunodeficiency virus (SIV) infection of non-human primates.

1.2 SIV

Like HIV-1, SIV is also a member of genus Lentivirus and the family *Retroviridae*. The SIV genome, however, is slightly bigger at about 10.6 kilobases. The SIV RNA genome, which is very similar to HIV-1, also consists of structural, accessory, and regulatory genes that encode for all the proteins used by the virus (Fig 3). One of the biggest structural differences between HIV-1 and SIV is in the accessory genes. HIV-1 contains *vpu* where many strains of SIV are missing this gene. A number of SIV strains instead have a duplication of *vpr* named *vpx*, where HIV-1 does not have *vpx* gene (Fig 3). As with HIV, there are a number of different SIV strains, which infect different primate species. Each of these strains has slightly different pathogenicity and tropism, but many general characteristics are similar.

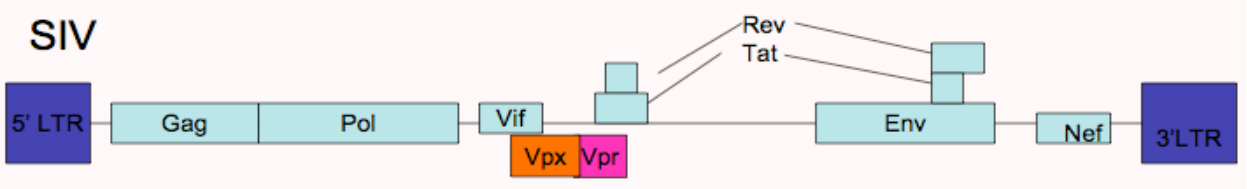


Figure 3. ***Genomic Organization of SIV***
Schematic depicting the organization of the SIV genome. Depicts all of the structural and non-structural genes in the virus. Shows difference between SIV and HIV is the deletion of Vpu and the addition of Vpx, which is not seen in HIV-1.

This project will mainly focus on the *vpx* and *vpr* genes, specifically in the SIVsmm strain. In SIVsmm, *vpx* and *vpr* have a high degree of homology, so much so that it is presumed that SIVsmm *vpx* originated from a duplication event of *vpr* [6]. Despite this, differences in function between SIVsmm *vpr* and *vpx* have been documented [5]. Additionally, while *vpx* is packaged within the virion, SIV *vpr* is produced *de novo* during productive infection [7, 8]. *Vpr* and *vpx* in SIVsmm and other SIV strains split the functions of HIV *vpr* (Table 2). Specifically, the gene

vpx appears to play a role in infection of non-dividing cells, viral replication, and efficient nuclear import of the pre-integration complex, while *vpr* is important in cell cycle arrest [5, 9, 10].

These differences between SIV and HIV should be studied in order to better interpret the roles of accessory genes and their interactions with the immune system. In order to accomplish this, in vivo studies must be performed. Before this can be done, however, in vitro studies must show that the effects of HIV-1 infection are similar to those of SIV infection. This project focused on this first step in developing a viable animal model for HIV-1 research. Observations made using HIV and HIV *vpr* mutants for infection were compared to the effects of SIV and SIV *vpr* and *vpx* mutants to determine which effects of HIV *vpr* were caused by SIV *vpr* and which were caused by SIV *vpx*. This project's results only touch briefly on the interaction of HIV and SIV with the host, begining the process of understanding this interaction and possibly leading to a more effective model for vaccine development.

1.3 DENDRITIC CELLS AND HIV

Dendritic cells (DCs) play a pivotal role in the generation of an effective immune response against incoming pathogens by transporting antigens from peripheral tissues to lymph nodes, where they are presented to circulating T-cells and B-cells, initiating T-cell and B-cell response [12]. The importance of DCs to the antiviral response leads many viral and bacterial pathogens to target DCs and T-cells [13-17]. Additionally, as part of their survival strategy, some microbial pathogens evade the host immune system by mimicking proteins involved in host defense. For example, poxviruses, cytomegalovirus, and herpesvirus encode homologue cytokine receptors, which can bind to the cognate cytokines and block their activities [23, 30].

While many different cell types are exposed to pathogens during the initial exposure of mucosal membranes, HIV-1 specifically targets immature DCs, Langerhan cells, and resting T-cells, which then promote viral replication and dissemination [19-23]. For example, immature DCs may be the first leukocytes to capture HIV-1 after it has crossed a mucosal surface, thereby transmitting virus to nearby CD4 T cells for amplification [5, 24-26]. Irrespective of the presence of viral antigens in infected antigen presenting cells (APCs), the immune response eventually fails to control HIV-1 virus production, creating an downward spiral that reduces the ability of DCs to induce antiviral immunity while simultaneously facilitating virus propagation. Indeed, while HIV-1-induced mutations leading to cytotoxic-T-lymphocyte (CTL) escape are thought to be the major cause of immune failure, recent studies have shown that accumulation of immature

dendritic cells in the periphery, in conjunction with CTL escape, could be part of HIV-1 progression, since these cells are then unable to effectively activate naive T-cells or B-cells [5, 27, 28].

Typically, DCs respond to pathogens by undergoing a well-regulated maturation process requiring the transcriptional regulation of both co-stimulatory molecules and cytokine-chemokine genes. These genes are necessary for antigen processing, presentation, and migration to lymphoid organs [29, 30]. Recent studies have demonstrated that DCs infected with HIV-1 selectively fail to undergo this maturation program, making them both incapable of activating lymphocytes as well as defective in interleukin-12 (IL-12) production [24, 31]. HIV-1 expresses a number of viral proteins that have been shown to dysregulate the host cellular immune response as part of immune evasive strategies. Exposure of DCs to *gp120* leads to an up-regulation of activation markers indicative of functional maturation [66]; *tat* inhibits antigen induced lymphocyte proliferation[24, 32]; the *nef* gene downregulates CD4 and major histocompatibility complex (MHC) class I molecules in T lymphocytes in order to escape CTL recognition [33, 34], and induces up-regulation of DC-specific ICAM-3 in DCs to facilitate viral spread [35]; and the Nef protein induces chemokines in primary macrophages that are thought to facilitate lymphocyte recruitment and activation [29, 35]. Though Tat and Nef are expressed early during infection, they are not packaged in the virus particle.

HIV-1 Vpr is a pleiotropic protein that is known to dysregulate a number of host cellular events, including cell cycle arrest, apoptosis, and host gene expression, upon its expression [37, 39, 40]. As it is present in the virion at detectable levels, it is one of the first HIV proteins seen by host

cells [36]. In patients with Vpr defects, disease progression, replication, and infection are attenuated, indicating that Vpr plays an important role in viral pathogenesis [38]. Specifically, the protein is necessary for the efficient infection of non-dividing cells, such as macrophages, and enhances viral replication within T-cell lines and activated peripheral blood lymphocytes [37]. Vpr is also known to persist in free and virion associated forms in vivo, exerting its effect on proximal and distal cells and tissues not actually infected by the virus [39]. In studies of HIV-1 Vpr's effect on DCs, Vpr has been shown to selectively impair the expression of co-stimulatory molecules and maturation markers, down-regulate the production and secretion of IL-12, and up-regulate IL-10, suggesting that Vpr plays a special role in altering the DC environment to impact maturation of these cells [41].

Like HIV-1 Vpr, SIV Vpx is also packaged in the virus particle, and is necessary for infection of macrophages [42, 43]. This, as well SIV Vpx's ability to promote replication and SIV Vpr's ability to lead to cell cycle arrest, suggests functional similarities between the two viruses and their effects on immune cells and pathogenesis. While the similarities between SIV *vpr* and *vpx* and HIV-1 *vpr* are being developed, many of the specific immune effects, such as the roles of these genes on DC maturation, have not been studied. Our goal in this study was to determine if the immune modulatory attributes of HIV-1 *vpr* are also seen with SIV *vpx* and *vpr*, and to determine whether SIV *vpr* or *vpx*, plays a role in this immune dysregulation.

1.4 NATURAL KILLER CELLS AND HIV

Natural killer (NK) cells are large granular lymphocytes that are critical for both innate and adaptive immune responses [44]. The immunologic role of NK cells has been shown in a variety of bacterial, fungal, and viral infections including HIV-1 [45, 46]. NK cells can lyse target cells by releasing cytokines, chemokines, and cytolytic granules containing perforins and granzymes in the absence of sensitization to antigens [47]. HIV-1 infection compromises both this innate and adaptive immunity and, unfortunately, antiretroviral drug treatment fails to fully restore NK cell function [48, 49]. In vivo studies indicate NK cells from viremic HIV-1 patients are defective in lysis of infected or target cells compared to aviremic or uninfected subjects [50, 51]. HIV-1 viral antigens have been shown to dysregulate NK cell function and its interaction with other accessory cells. Thus the dysregulation of NK cell phenotypes, together with compromised cytolytic function, likely contributes to the loss of natural immunity during HIV-1 infection [44, 52, 53].

Structural and accessory proteins of HIV-1 are known to interact with components of both innate and adaptive immune systems to evade host immune surveillance [54, 55]. HIV-1 Nef is a potential regulator of NK cell cytotoxicity by MHC class I down regulation in infected $CD4^+$ T cells [56]. HIV-1 Tat has been shown to inhibit NK cell function through inhibition of LFA-I-mediated Ca^{2+} influx [57]. HIV-1 envelope also inhibits NK cell cytotoxicity and survival [58-60]. From these studies it is evident that HIV-1 uses several of its gene products to disrupt an early line of defense against infection.

HIV-1 Vpr is associated with the virus particles, and is available to host cells prior to de novo synthesis and productive infection [39], suggesting that Vpr might have a role in the dysregulation of innate immunity and NK cell dysfunction. Results published by Majumder *et al* indicate that NK cells present in PBMC infected with HIV-1 Vpr (+) virus are defective in their ability to lyse NK-sensitive targets and in production of the degranulation marker, CD107a, and thus suggest a role for HIV-1 Vpr in compromising NK cell function and innate immunity [61].

Similar studies in SIV infected rhesus monkeys to determine if any dysregulation of NK cells occurs found that within infected monkeys, NK cells were impaired in their production of IFN-γ, TNF-α, and IL-2, as well as down-regulation of NK activating molecules, NKG2C and NKG2C2 [62]. Our study aims to show that, along with these receptor and activation molecule changes, SIV *vpr* and/or *vpx* also plays a role in inhibiting the functional capacity of NK cells, through down-regulation of degranulation markers and disruption of the subset distribution. Subset distribution is the number of cells that are active and able to lyse versus the number of anergic cells that are no longer capable of acting on infected cells.

1.5 SPECIFIC AIMS

HIV-1 Vpr is shown to regulate both viral and host cellular functions thereby enhancing the viral replication as well as evading the host immune system. This includes inhibition of cell proliferation, induction of apoptosis, translocation of PIC into the nucleus of non dividing cells, immune evasion and host cellular dysregulation. Most of the Vpr-related functions are mediated by virion associated Vpr exposure during early infection similar to de novo synthesized Vpr. In case of SIV, Vpr has a duplication, Vpx with high homology indicating that both SIV Vpr and Vpx might be involved in similar host cellular events. However, Vpx is packaged as virion associated molecule whereas Vpr is not. Based on these we hypothesis that cellular events regulated by virion associated Vpr via virus uptake might be regulated by SIV Vpx, whereas functions regulated primarily by de novo synthesized Vpr might be mediated by SIV Vpr. The aims of my project are to delineate the functions of SIV Vpx and SIV Vpr using virus exposed and infected cell systems and mutant SIV viruses.

Specific Aim 1:

- To determine the effects of SIV Vpr and Vpx on DC maturation and cytokine production (adaptive immune response)
    - Differentiate between the virus exposed and unexposed cells
    - DC functional assays to determine surface marker changes in the presence of virus
    - Cytokine analysis to determine changes in the key cytokines that drive the immune response

Specific Aim 2:

- To determine the effects of SIV Vpr and Vpx on NK cell function (innate immune response)
    - Functional analysis of NK cells within the infected milieu
    - NK subset distribution changes in the presence of mutated virus

2.0 MATERIALS AND METHODS

2.1.1 Cell culture

Monocyte-derived dendritic cells were generated from peripheral blood mononuclear cells (PBMCs). Heparinized blood samples were obtained with written consent from normal, healthy donors. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. $CD14^+$ monocytes were purified by positive selection using anti-CD14 monoclonal antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA). Purity of $CD14^+$ cells was tested by flow cytometry using CD14-PE (BD-Pharmingen, San Diego, CA) and CD1a-FITC (ImmunoTech, Miami, FL) antibody. Greater than 95% of isolated cells were CD14 positive (characteristic phenotype of myeloid derived monocytes) at day 0 of culture and 85% CD1a positive (characteristic of immature Dendritic Cells) at day 7 of culture. To obtain monocyte-derived DC, $CD14^+$ cells ($0.5X10^6$ cells/ml) were cultured in 60 mm culture plates in a total volume of 10 mL RPMI (GIBCO, Gaithersburg, MD) media containing 10% FBS, 1% L-glutamine (Cambrex, Walkersville, MD), 1% penicillin-streptomycin (GIBCO), 25 ng/ml IL-4 (R&D Systems, Minneapolis, MN) and 50 ng/ml GM-CSF (R&D Systems). Half the volume of media was replaced every third day with fresh media containing IL-4 and GM-CSF throughout the entire culture period.

PBMCs isolated by Ficoll-Hypaque gradient centrifugation were cultured for 2 days in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and rIL-2 (5 U/ml).

HEK293T cells were maintained in DMEM containing 10% FBS, 1% L-glutamine (Cambrex) and 1% penicillin-streptomycin (GIBCO).

K562 cells were maintained in RPMI containing 10% FBS, 1% L-glutamine (Cambrex) and 1% penicillin-streptomycin (GIBCO).

2.1.2 Proviral Constructs

SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, and SIV PBjΔVprΔVpx proviral clones were made and kindly provided by Dr. Fletcher's group by site directed mutagenesis, as described [7] (Fig 4). Each construct was transformed into JM109 strain of *E. coli* bacteria and grown on agar plates. Colonies were then screened by gel electrophoresis and sequencing for the correct clones with the correct mutations in the proper positions.

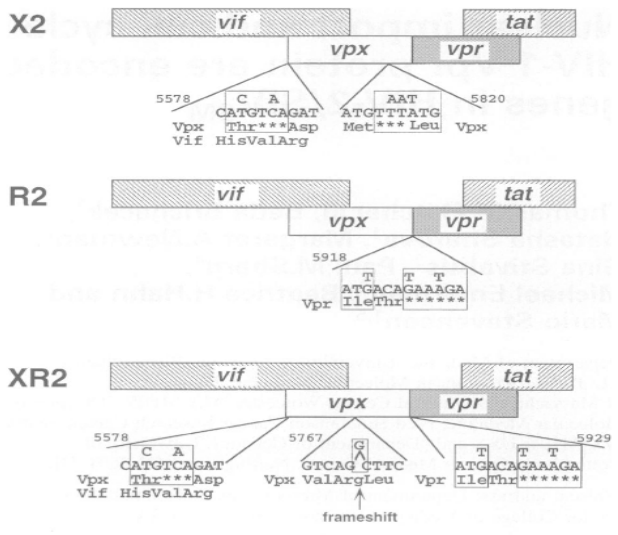


Figure 4. *SIV PBj Vpr and Vpx mutants*
Figure taken from Fletcher *et al* [7], used with permission, describing the construction of the mutants used in this study. Site directed mutagenesis was used to place stop-codons in to prevent the synthesis of the protein. X2 is the *vpx* mutant, R2 is the *vpr* mutant, and XR2 is the double mutant for both *vpr* and *vpx*. All proviral constructs were a gift from Dr. Vanessa Hirsch, NIH. Along with these mutants a wild-type proviral clone was also used.

2.1.3 Virus Preparation

SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, and SIV PBjΔVprΔVpx viral constructs were pseudotyped with vesicular stomatitis virus (VSV-G) envelope protein, denoted as WT, ΔVpr, ΔVpx, and ΔVprΔVpx respectively. HEK293T cells ($2x10^6$) were co-transfected with 7.5µg of SIV PBj proviral construct (SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx) and 2.5µg VSV-Glycoprotein-Envelope (VSV-G-Env) expression plasmids by the calcium phosphate precipitation method. Forty-eight hours post-transfection, supernatants were collected, spun at 3000 rpm and filtered through a 0.4µm filter to remove cellular debris. All virus stocks were further concentrated by ultra centrifugation at 22,000 rpm for 1 hour at 4^oC. Virus pellets were resuspended in PBS and stored at –80 C for subsequent assays. Virus titer was measured using a p27 ELISA kit and associated protocol (Zeptometrix).

2.1.4 Virus Staining

In initial experiments SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, and SIV PBjΔVprΔVpx pseudotyped viruses were stained directly with Cy5 mono-reactive dye (Amersham). 1 mg/ml of virus was used for the staining reaction. Virus sample dissolved in 1mL of 0.1 M sodium carbonate-sodium bicarbonate buffer and then added to the dye vial. This was mixed thoroughly by inverting the vial. The reaction was incubated at room temperature for 30 minutes, mixing every 10 minutes. Labeled virus was separated from free dye through the use of a G-25 M Sephadex column (Amersham). The column was equilibrated with DPBS twice before adding the reaction mix. The reaction mix was added to the column and further DPBS was added to the column to allow the mix to run through the column. Two bands can be seen on the column, the faster moving band is the labeled virus, while the slower moving band is the free dye. The first band was collected, made into small aliquots and stored at -20C. Virus titer was measured by p27 ELISA kit and associated protocol (Zeptometrix).

2.1.5 Immunophenotypic analysis of SIV PBj virus exposed dendritic cells

DC, after 6 days of culture with IL-4 and GM-CSF, were exposed to 100ng of SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx virus for 48 hours, were stimulated with or without 1 μg/ml lipopolysaccharide (LPS) (Sigma Aldrich) for final 24 hours. Expression of DC phenotypic maturation markers (CCR7, CD83, CD86, and HLADR) were detected both in unexposed and SIV PBj exposed cells using fluorescently conjugated monoclonal antibodies. The antibody concentrations used in this experiment were as per the manufacturer's suggestions (CCR7: R and D cat# FAB197F, CD83: Beckman Coulter cat# PN 1M2218U, CD86: Beckman

Coulter cat# PN IM2729, and HLADR: BD Bioscience cat# 555813). This was followed, in initial experiments by permeablization of the cells using BD CytoFix/Perm solution for 20 minutes followed by two washes (BD). Cells were incubated with 5µl of p27 antibody for 15 minutes and then washed twice (Advanced Biotechnologies; cat# 13-113-100). A FITC labeled anti-strepavidin secondary antibody was incubated with the cells for another 15 minutes and then washed twice. Cells were then resuspended in DPBS with 3% FBS.

2.1.6 Biotinylation of p27 antibody used in flow cytometry

The p27 Gag protein specific antibody (Advanced Biotechnologies; cat# 13-113-100) was biotinylated using a 25mg/ml solution of Biotin long arm NHS (Vector Labs cat. #SP-1200) made in DMSO. Of this solution, 1µl (25µg) was added to a vial of antibody (100µl), and the tube was mixed well and incubated for 3 hours at room temperature, mixing periodically. The antibody was then transferred to a small dialysis cassette (Slide-A-lyzer; Pierce; 2500m.w. cutoff), placed in a floating rack, and dialyzed in 1L of PBS with a slow stirbar for 1 hour. This was repeated with 2 fresh liters of PBS, and the last wash was overnight at 4^{o}C. The antibody was then collected and stored at 4^{o}C.

2.1.7 Measurement of TNF-α, IL-12, IL-10, and IL-15 by ELISA

Following stimulation, supernatants were collected and analyzed for the presence of TNF-α (BD Bioscience cat# 555212), IL-12 (BD Bioscience cat# 555183), IL-10 (BD Bioscience cat# 555157), and IL-15 (BD Bioscience cat# 559268) using Opti-EIA ELISA assay kits according to

the manufacturer's protocol. ELISA plates were developed using the TMB Substrate Reagent Set (BD Bioscience cat# 555214).

2.1.8 Western Blot

Uninfected and Cy5 labeled SIV PBj WT virus exposed DC at varying concentrations were washed twice with PBS and lysed in RIPA buffer containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton-X100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1.0mM phenylmethyl-sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin inhibitor units/ml aprotinin, and protease inhibitors leupeptin, chymostatin, and pepstatin (1 μg/ml; Sigma). Cell lysates were clarified by centrifugation and total cell lysates (50μg) were separated on a SDS-PAGE gel, transferred, and the expression of p27 gag protein (Advanced Biotechnologies cat# 13-113-100) was detected. Loading was based on protein estimation prior to loading.

Western Blots were also done on virus pellets to determine the reactivity of the p27 gag protein antibody (Advanced Biotechnologies cat# 13-113-100). The pellets were separated on a SDS-PAGE gel, transferred, and the expression of p27 gag protein was detected.

2.1.9 NK Cell Degranulation and Subset Distribution Assay

To quantitate the cell surface expression of CD107a and determine the subset distribution of NK cells, infected PBMC (total $1X10^6$/mL) were washed twice in PBS, and activated with K562 target cells (PBMC: K562=1:1) in a total volume of 200μL pre-warmed culture media following centrifugation for 5 minutes at 1200 rpm to facilitate the contact of cells. PBMCs stimulated for

one hr with K562 cells were further incubated with 6µl of Golgistop (BD Bioscience, Mountainview, CA) at a final concentration of 6µg/ml for an additional three hrs. Cells were also stained with 10µL of anti-CD107a-FITC antibody for the entire duration of the incubation. Cells were collected and washed with PBS containing 3% FBS. Cells were then stained with an additional 10 µl per tube of anti-CD107a-FITC antibody (BD Bioscience cat# 555800) as well as with anti-CD3-ECD antibody (Beckman Coulter cat# PN IM2705U), anti-CD16-PC5 antibody (BD Bioscience cat# 555408), and anti-CD56-PE antibody (Beckman Coulter cat# PN IM2073), for surface expression and determination of subset distribution of NK cells, for additional one hour in ice. Cells were washed with PBS containing 3% FBS two times and then resuspended in 400µL of wash buffer. Cells were read by flow cytometry by gating on CD3- and CD56+ NK cells and those cells were then evaluated for CD107a and CD16 expression.

2.1.10 Statistical analysis

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

3.0 RESULTS

3.1 THE EFFECTS OF SIV VPR AND VPX ON DC MATURATION AND CYTOKINE PRODUCTION

3.1.1 Differentiating Between Virus Exposed and Unexposed Dendritic Cells

In order to properly evaluate the effects of virus exposure, it is essential to differentiate between virus exposed and unexposed cells. A p27 antibody was utilized to attempt to identify DCs transduced with the virus. SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx virus pellets, collected 72 hours post transfection and concentrated using high speed centrifugation, were examined by western blot to insure that the antibody could indeed detect p27 in the virus treated samples. Results from this attempt indicated that the antibody detected p27 in each of the virus treated cultures and not in the cultures not treated with virus, showing the antibody was specific and could detect p27 in cultures treated with each of the virus types (Fig 5).

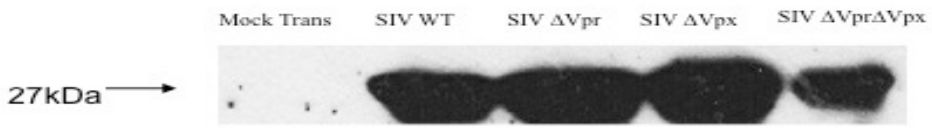


Figure 5. ***p27 Staining of SIV Virus Pellet***
Western blot using anti-p27 specific antibody to detect virus particle in supernatants collected from transfected 293T cells. Mock trans is supernatant from 293T cells that were transfected with irrelevant plasmid vector control.

Because the antibody was effective in detecting Gag (p27) in virus treated samples, and these levels correlated with the levels of p27 determined in the virus pellet by the p27 ELISA kit, it confirmed the antibodies ability to detect the protein and we decided to use it to detect p27 through flow cytometry. This antibody presented a possible method of differentiating between virus exposed and unexposed cells. The antibody was then biotinylated for use with a FITC fluorochrome conjugated secondary antibody for use in flow cytometry. Immature monocytes derived dendritic cells (iMDDC) were then exposed SIV PBj WT virus at a concentration of 100ng of p27, as determined by p27 ELISA. The cells were then collected and stained with the anti-p27 primary antibody and the FITC conjugated secondary antibody at varying concentrations. The cells were then screened by flow cytometry to determine which concentration was best able to differentiate between the virus exposed and the unexposed DCs. It was determined that at low concentrations the antibody did not stain virus treated cells, and at higher concentrations the antibody bound non-specifically to the cells in both the treated and the untreated samples (Fig 6). This non-specific binding was not seen in the IgG control antibody but suggesting this was a problem with the p27 antibody specifically (Fig 6).

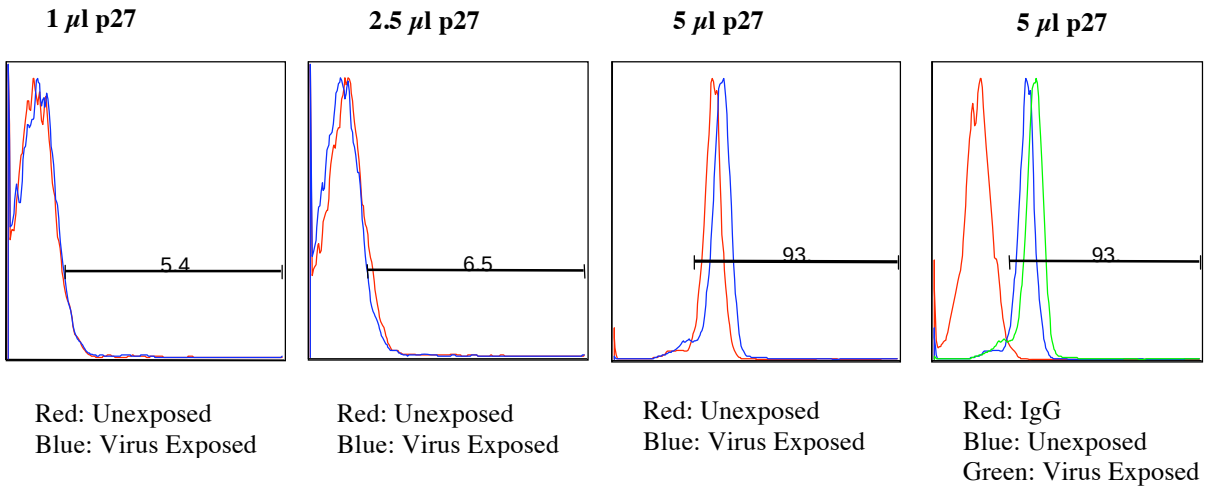


Figure 6. *p27 Staining of Immature Dendritic cells*
Immature dendritic cells were exposed to virus and were then stained with varying concentrations of anti-p27 antibody. The stained samples were then evaluated by flow cytometry.

These results suggested that there was either a problem with the use of this p27 antibody in flow cytometry in general or in DCs. We tested the antibody in PBMCs in order to determine the reason for the problem. PBMCs were used after infection with SIV PBj WT for 3, 6, and 9 days. Each day samples were collected and stained with differing concentrations of p27 antibody and analyzed by flow cytometry. It was determined that at a concentration of 5µl of antibody, it was possible to detect p27 in the virus exposed cells, and this was specific to the virus exposure as no p27 was detected in the unexposed cells (Fig 7).

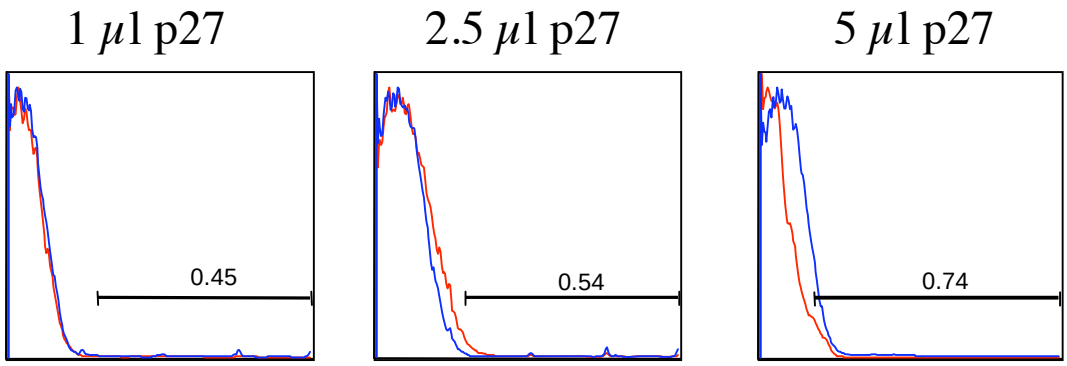


Figure 7. *p27 Staining of PBMCs*
Human PBMCs were exposed to SIV PBj WT and were then stained with p27 antibody at differing concentrations to determine what concentration showed the best detection ability.

The percentage of p27 positive cells also increased on day 6 and day 9 suggesting an increase in infected cells, as would be expected (Fig 8).

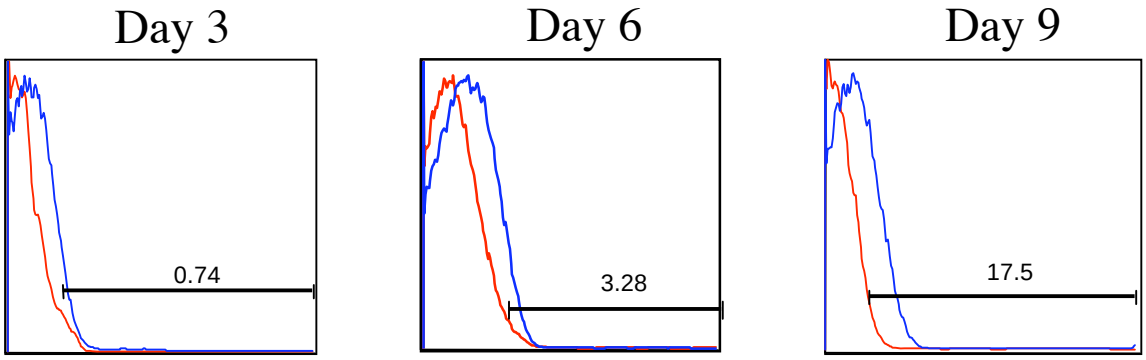


Figure 8. ***PBMCs exposed to SIV PBj WT Virus Over Time***
PBMCs were exposed to SIV WT virus over 3, 6, or 9 days and were then harvested and stained with 5µl of anti-p27 antibody and were then evaluated by flow cytometry for changes in the number of p27 positive cells.

This suggested that the problem was not with the p27 antibody, but instead was a problem with the use of this antibody in DCs, and that within DCs the antibody bound non-specifically and would not be useful in differentiating between virus-exposed and unexposed DCs.

An alternative to p27 staining was examined. Instead of exposing cells to virus and looking for the cells that had picked up virus by antibody staining, the virus itself was tagged with a fluorochrome and this tagged virus was then used to infect the DCs. Flow cytometry was then used to follow the tagged virus and distinguished between cells that took up virus and those that did not. Initial studies were done using only SIV PBj WT virus tagged with the Cy-5 fluorochrome, to determine whether the tagged virus was visible using flow cytometry. Dendritic cells were infected with increasing amounts of virus and the cells were evaluated by flow cytometry. The amount of tagged virus picked up by the DCs increased as the concentration of virus increased, as seen by the increase in MFI (Fig 9).

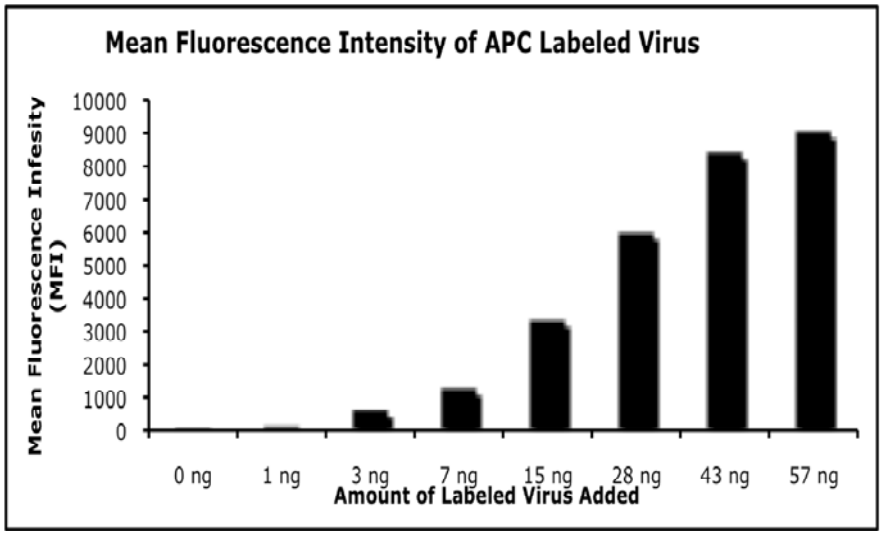


Figure 9. ***Dendritic Cells Exposed to Cy5 Labeled Virus***
Immature dendritic cells were exposed to Cy5 labeled virus for 72 hours at varying concentrations based on p27 ELISA measurements. Cells were collected and fixed and evaluated by flow cytometry. Mean fluorescence was determined by the use of FLOWJO software.

This showed that, at increasing concentrations, we were able to see the virus being taken up by the cells and the virus was tagged appropriately. We then looked at the percent of cells positive for the virus, in order to see the DCs that took up virus versus those cells that did not. This was also done using flow cytometry and it was seen that there was actually no cell population that did not pick up some virus at virus concentrations typically used (Fig 10). This suggested that all dendritic cells are exposed to the virus, even at low concentrations. Due to these results the remaining experiments used unstained virus for simplicity.

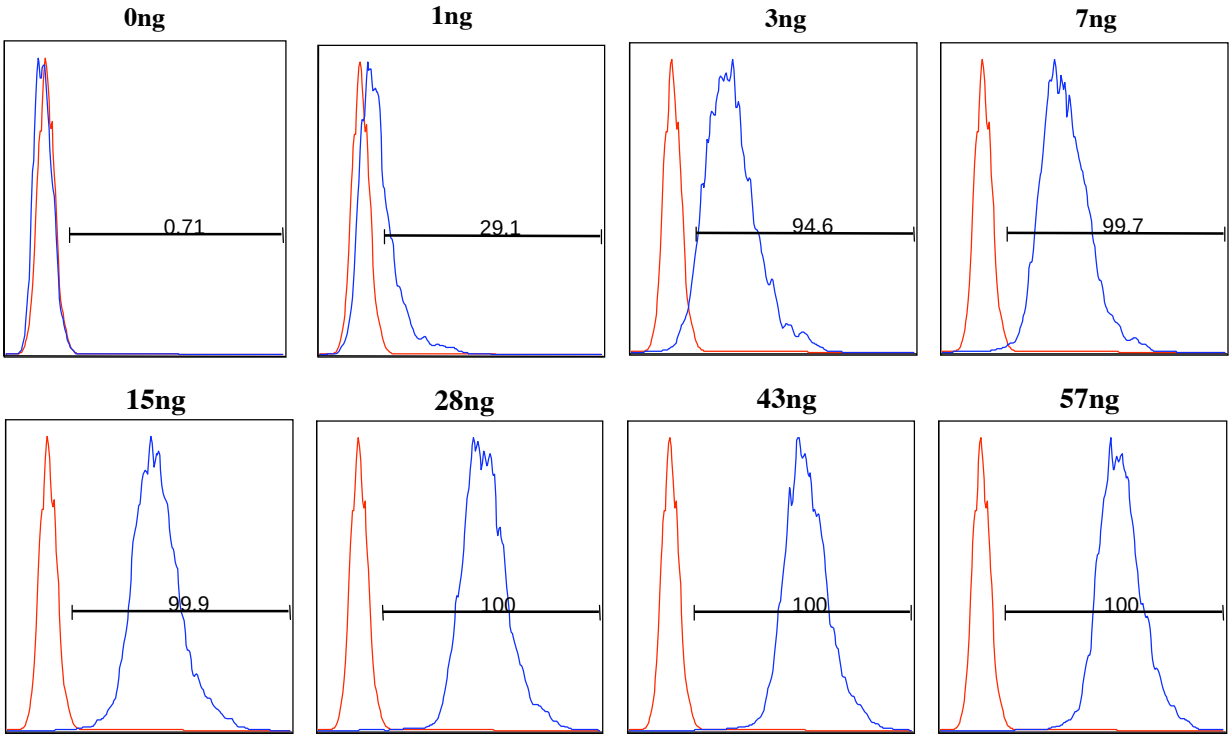


Figure 10. ***Immature Dendritic Cells Exposed to Cy-5 tagged SIV PBj WT***
Immature dendritic cells exposed to APC-labeled SIV PBj WT virus at varying concentrations. Histograms show the percent of total cells expressing APC, suggesting uptake of or exposure to virus.

3.1.2 Determining Effect of SIV PBj Mutant Viruses on Dendritic Cell Surface Marker Expression

We looked at important maturation markers such as CD83 and CD86, as well as migration marker, CCR7, which tells the DC to move to the lymph node, and HLA-DR, a control to ensure the up or down regulation seen was specific and not an up or down regulation of all surface markers. Initial studies were done comparing tagged and untagged virus to determine whether the changes could be seen without differentiating between cells that took up virus. Stained and unstained SIV PBj WT virus was used in this experiment and the cells were stained for CD83, CD86, and CCR7 (Fig 11-13). It was found that these results were similar to those seen with the unstained virus, suggesting again that all cells pick up virus, and the surface marker changes/differences could be seen without having to establish separate populations of cells that picked up virus and those that did not (Fig 11-13). The labeled and unlabeled viruses were run on separate machines, but the percentages were comparative suggesting the populations are comparable.

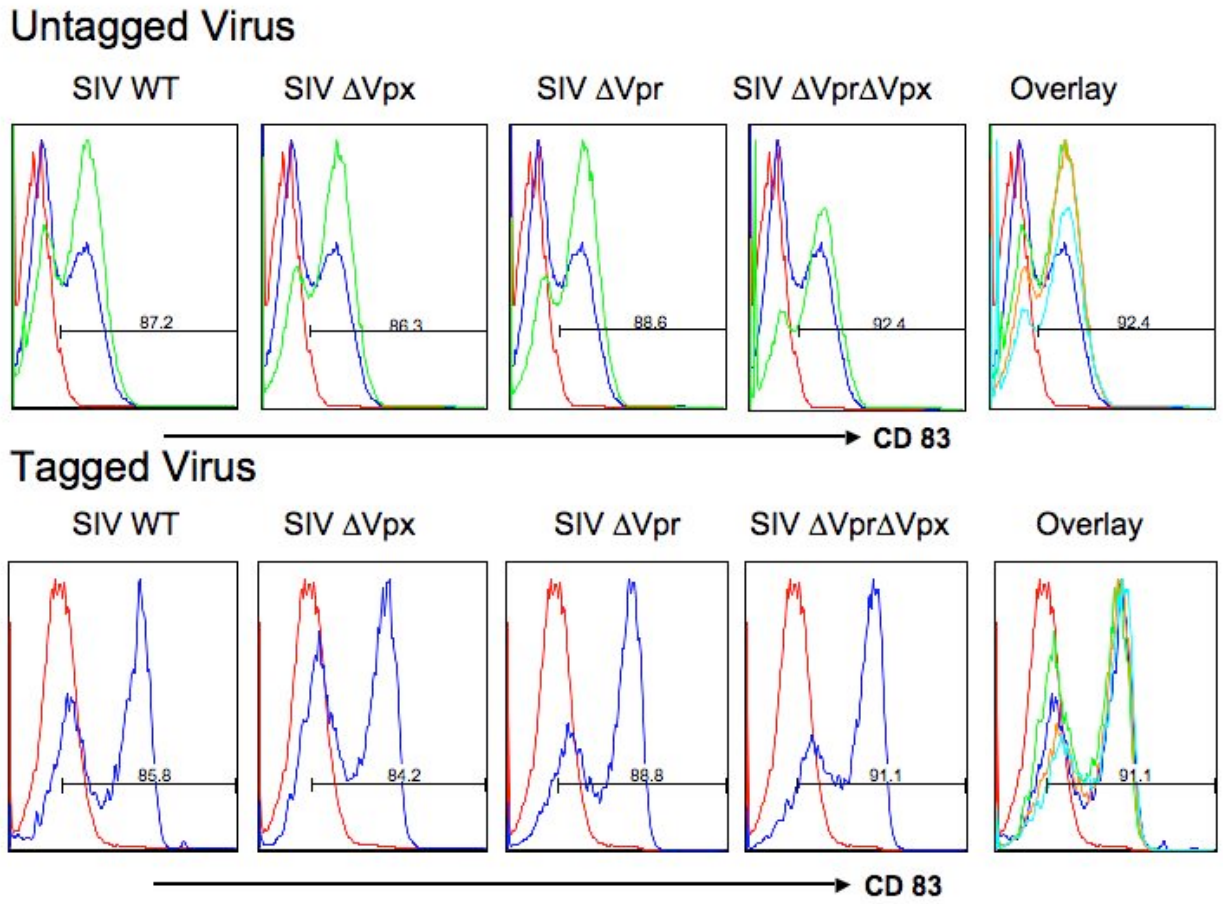


Figure 11. ***Comparing Labeled and Unlabeled Virus Exposed Cells for CD83 Expression***
One set of immature dendritic cells was exposed to APC tagged virus and another set of immature dendritic cells was exposed to untagged virus at the same concentrations. Donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Untagged virus was prepared similarly but without the Cy5 label. Cells were then collected and fixed and stained for the maturation marker CD83. They were then evaluated by flow cytometry for expression of these markers. For the untagged virus, the red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells. For the tagged virus, the red histograms are the unexposed cells and the blue are the virus-exposed cells. Gating was set on based on the IgG control.

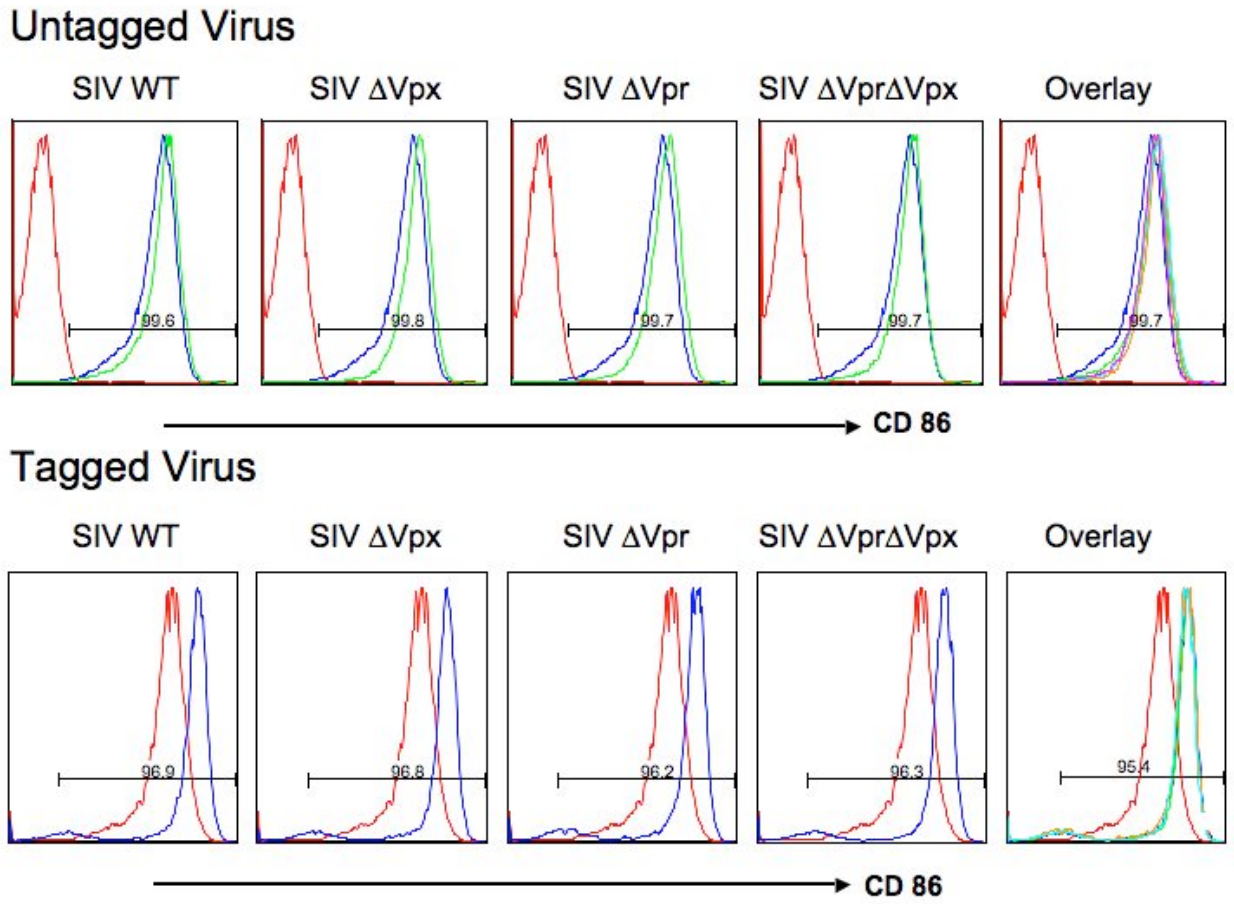


Figure 12. ***Comparing Labeled and Unlabeled Virus Exposed Cells for CD86 Expression***
One set of immature dendritic cells was exposed to APC tagged virus and another set of immature dendritic cells was exposed to untagged virus at the same concentrations. Donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Cells were then collected and fixed and stained for the maturation marker CD86. They were then evaluated by flow cytometry for expression of these markers. For the untagged virus, the red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells. For the tagged virus, the red histograms are the unexposed cells and the blue are the virus-exposed cells. Gating was set on based on the IgG control.

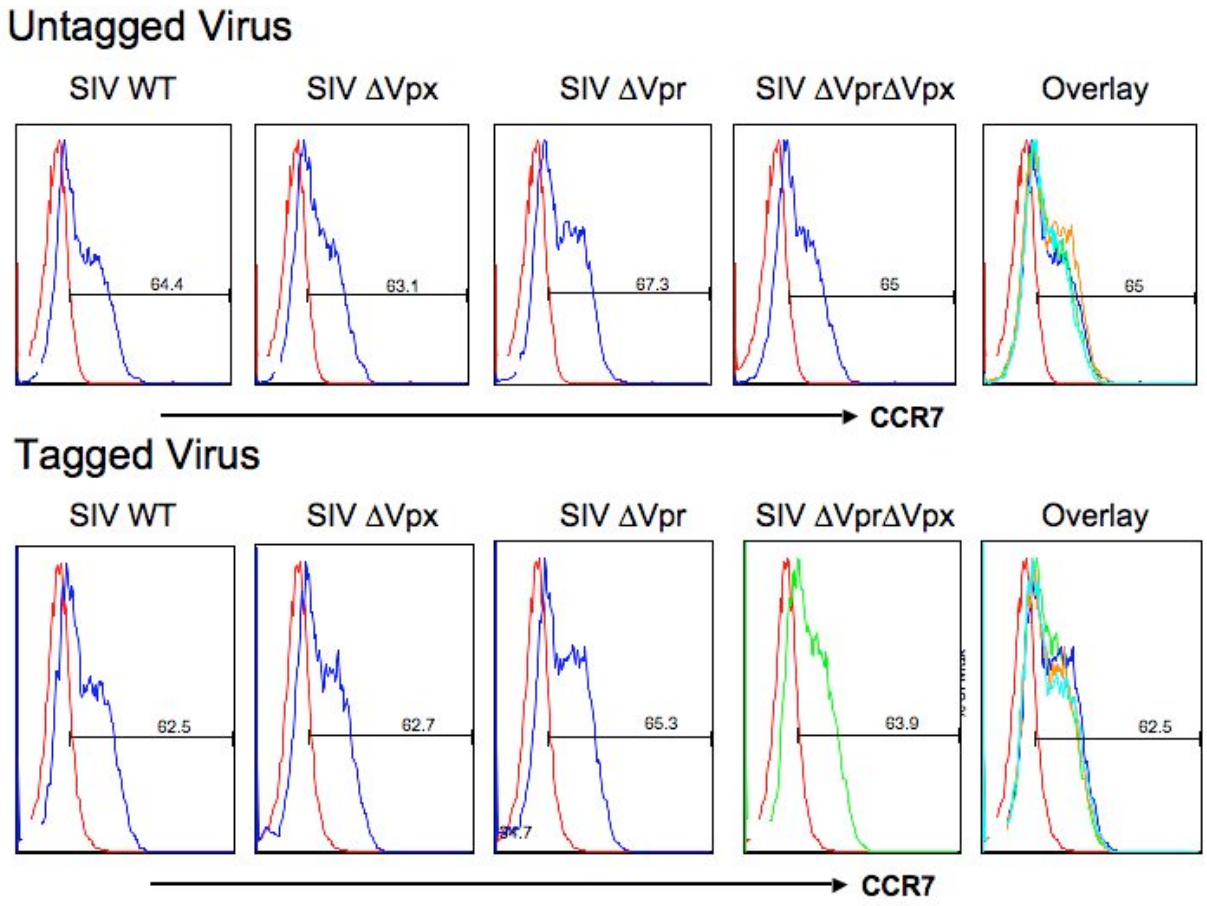


Figure 13. ***Comparing Labeled and Unlabeled Virus Exposed Cells for CCR7 Expression***
One set of immature dendritic cells was exposed to APC tagged virus and another set of immature dendritic cells was exposed to untagged virus at the same concentrations. Donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Cells were then collected and fixed and stained for the migration marker CCR7. They were then evaluated by flow cytometry for expression of these markers. For the untagged virus, the red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells. For the tagged virus, the red histograms are the unexposed cells and the blue are the virus-exposed cells. Gating was set on based on the IgG control.

Once it was determined that nearly all DCs pick up virus after exposure, we then went on to look at the effect of this virus exposure on the maturation of these immature DCs with unlabeled virus. We again looked at CD83 and CD86, as well as CCR7. The presence of these makers in six donors was tested by 3-color flow cytometry. Compensation was done with singly stained DCs from the respective donor, along with doubly stained cells to ensure that each color fell in

the appropriate quadrant. Each donor had an unstimulated, LPS, and/or CD40L stimulated group. Each donor was treated the same way with the addition of 100ng of each virus. Donor variability provided some differences in the response of each donor, but a representative donor, donor 4, shows the general trend of the response in each (Fig 14-19). As also seen in the flow cytometric data, the change in the surface marker expression was specific to these maturation and migration markers, as HLA-DR expression remained the same regardless of treatment or stimulation.

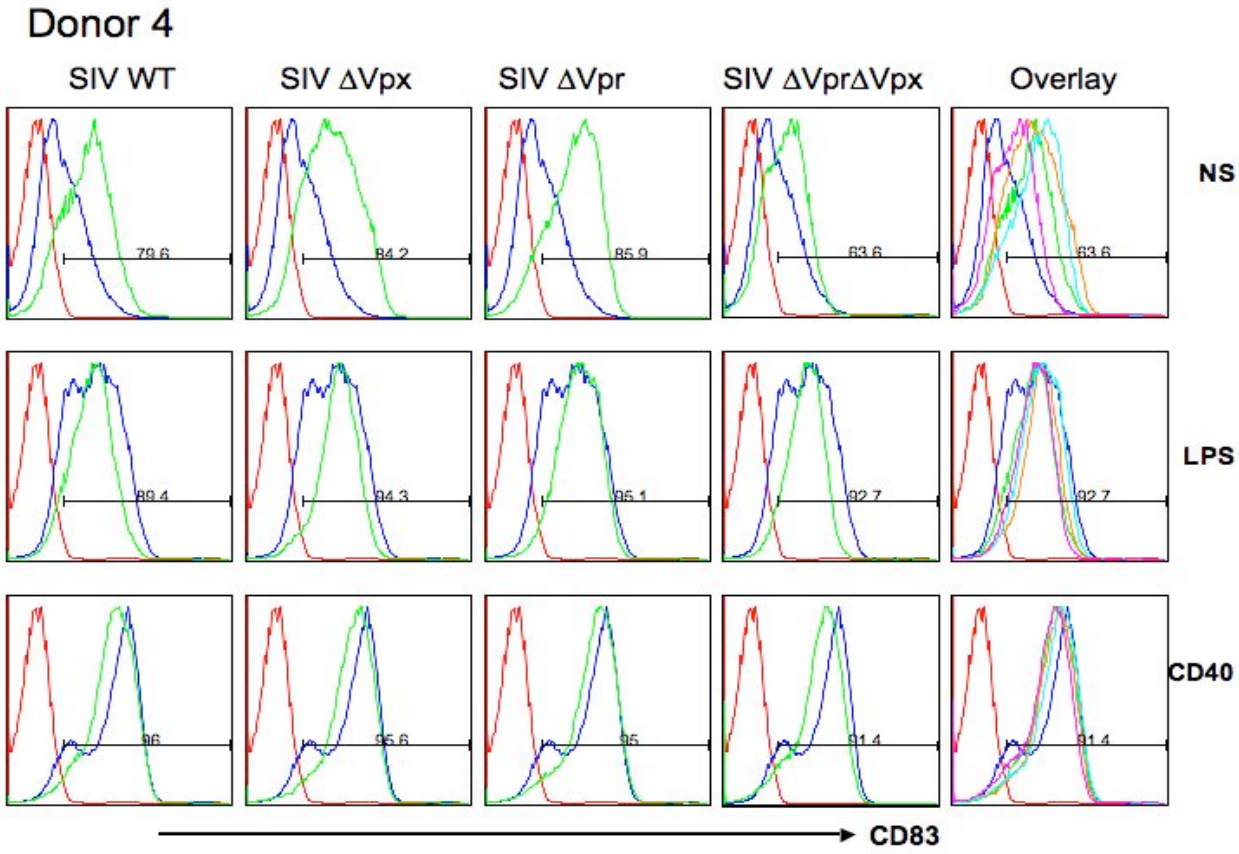


Figure 14. ***Representative Donor for CD83 Surface Maker Expression***
Six donors were exposed to each virus type, either SIV WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx. These were then either left unstimulated or were stimulated with CD40L or LPS for 24 hours. Cells were then collected, stained for the above surface maturation markers, and fixed. Cells were then evaluated by flow cytometry. This plot is representative of the 6 donors. The red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells.

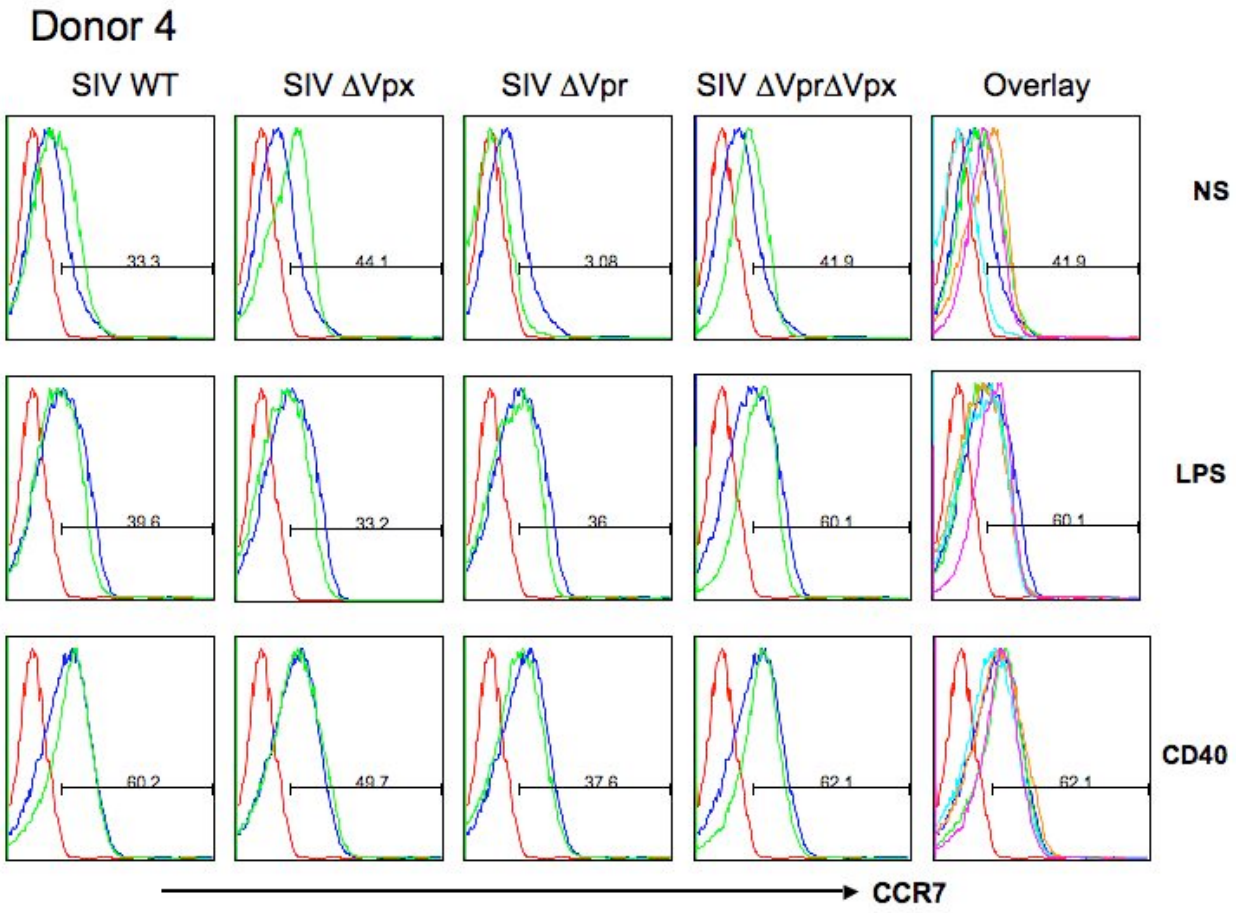


Figure 15. ***Representative Donor for CCR7 Surface Maker Expression***
Six donors were exposed to each virus type, either SIV WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx. These were then either left unstimulated or were stimulated with CD40L or LPS for 24 hours. Cells were then collected, stained for the above surface maturation markers, and fixed. Cells were then evaluated by flow cytometry. This plot is representative of the 6 donors. The red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells.

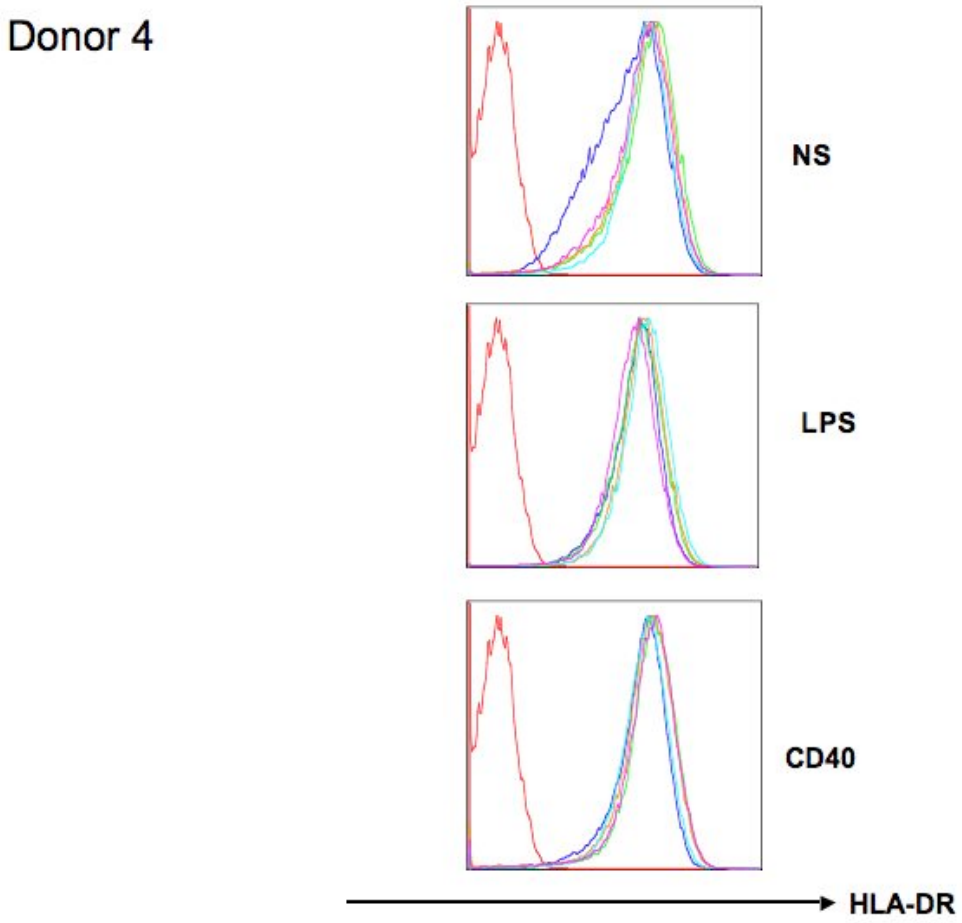


Figure 16. ***Representative Donor for HLA-DR Surface Maker Expression***
Six donors were exposed to each virus type, SIV WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx. These were then either left unstimulated or were stimulated with CD40L or LPS for 24 hours. Cells were then collected, stained for the above surface maturation markers, and fixed. Cells were then evaluated by flow cytometry. This plot is representative of the 6 donors. The red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells.

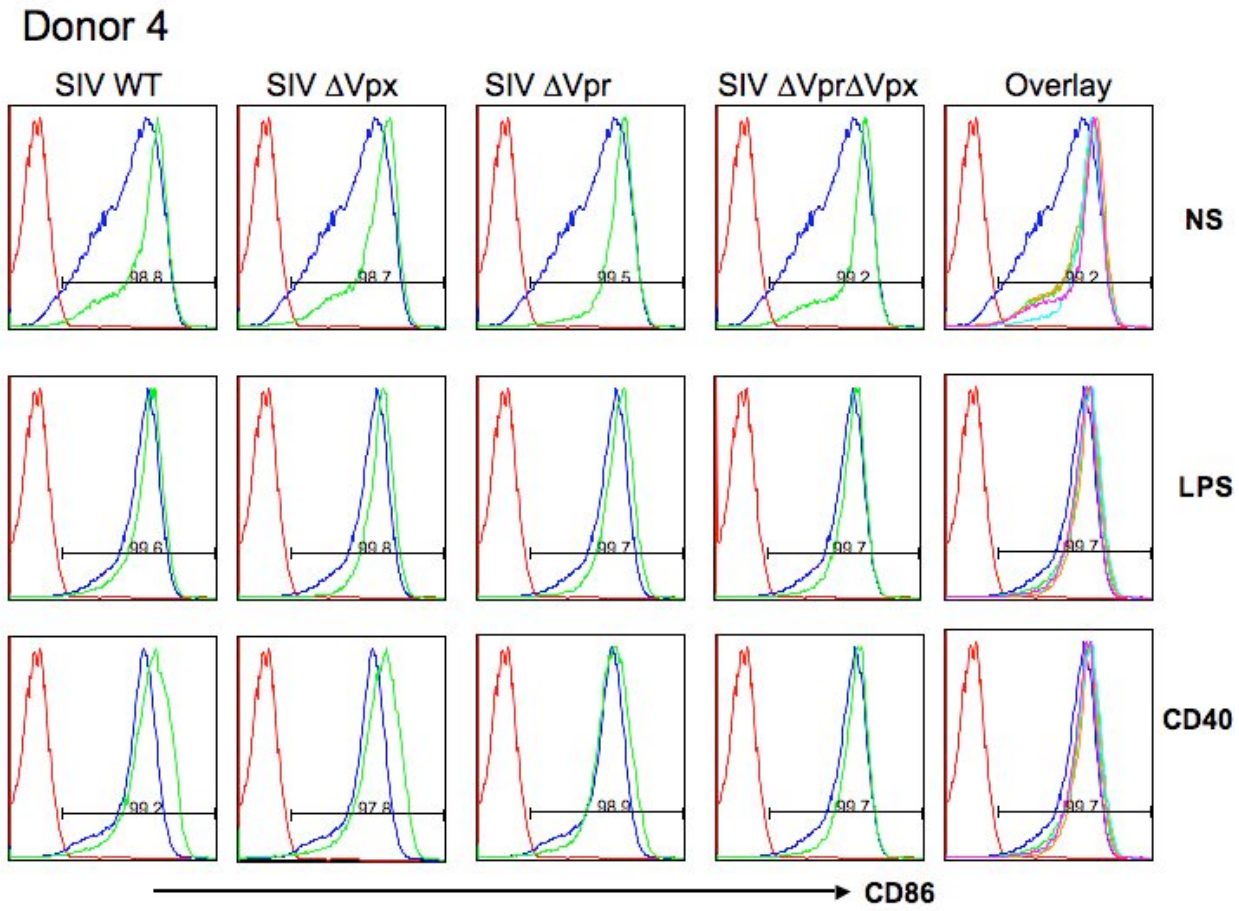


Figure 17. ***Representative Donor for CD86 Surface Maker Expression***
Six donors were exposed to each virus type either SIV WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx. These were then either left unstimulated or were stimulated with CD40L or LPS for 24 hours. Cells were then collected, stained for the above surface maturation markers, and fixed. Cells were then evaluated by flow cytometry. This plot is representative of the 6 donors. The red histograms are the IgG control, blue are the unexposed cells, and green are the virus-exposed cells.

Along with the percent of cells we also assessed the mean fluorescence intensity, MFI, or the amount of each molecule present on the cell surface (Fig 18-20).

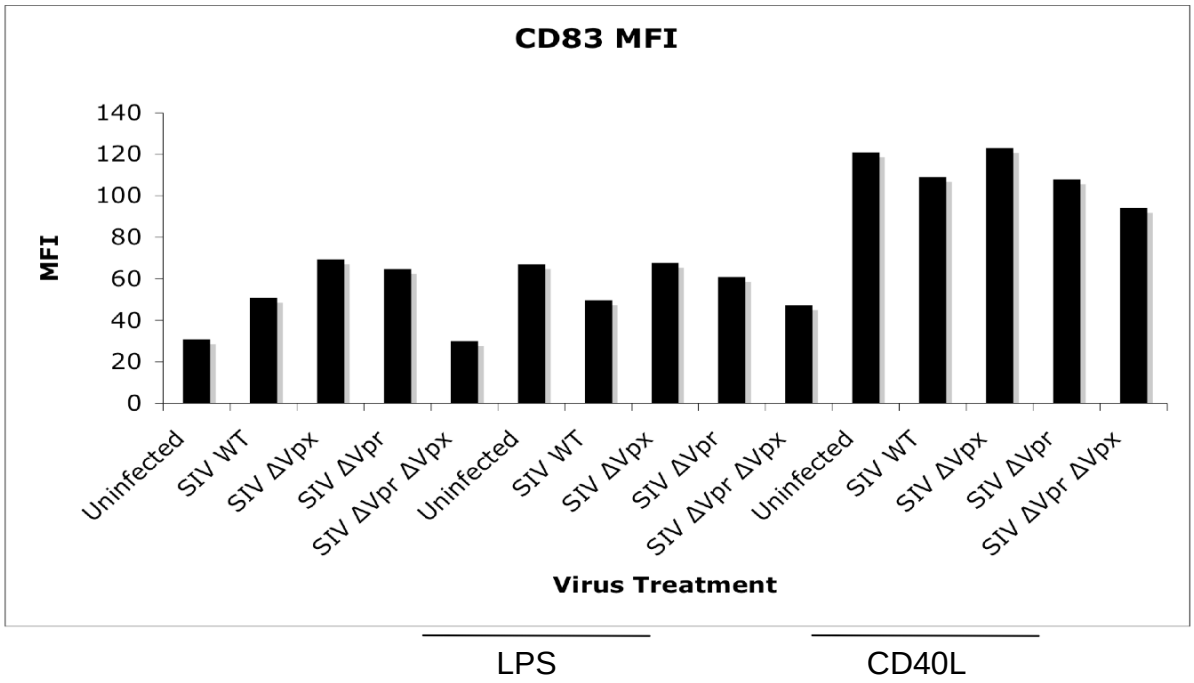


Figure 18. ***CD83 MFI Data for One Donor***
Represents the mean fluorescence intensity of CD83 for one representative donor. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Data was gathered by flow cytometry and software analysis by FLOWJO.

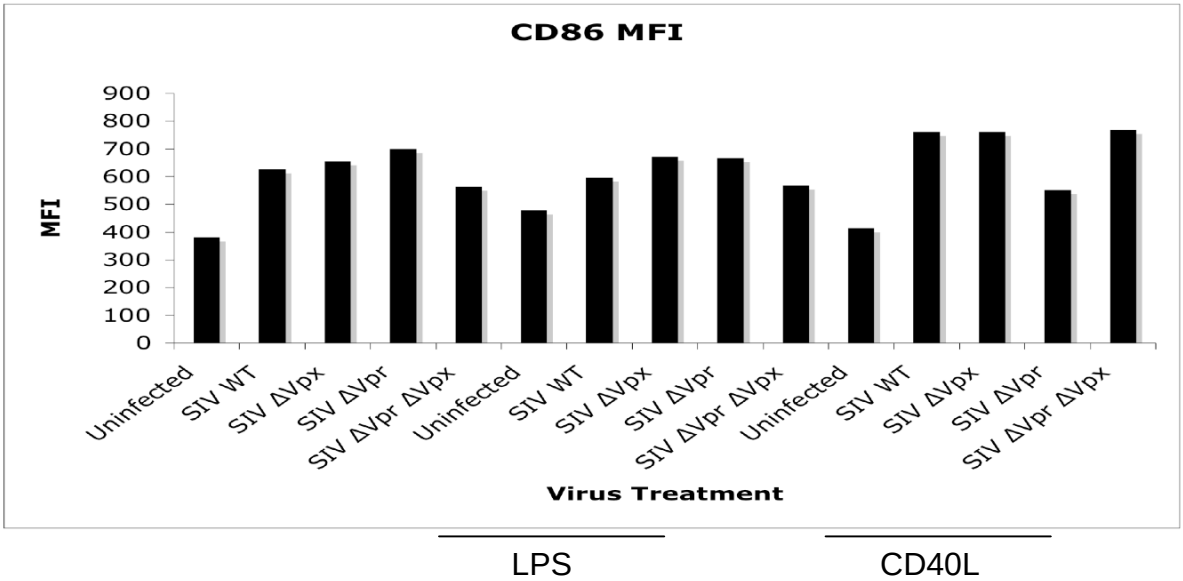


Figure 19. ***CD86 MFI Data for One Donor***
Represents the mean fluorescence intensity of CD86 for one donor with similar results as compared to other donors. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Data was gathered by flow cytometry and software analysis by FLOWJO. Cells were either left unstimulated, as in the first 5 bars, or stimulated with LPS, as in the middle 5 bars, or stimulated with CD40L.

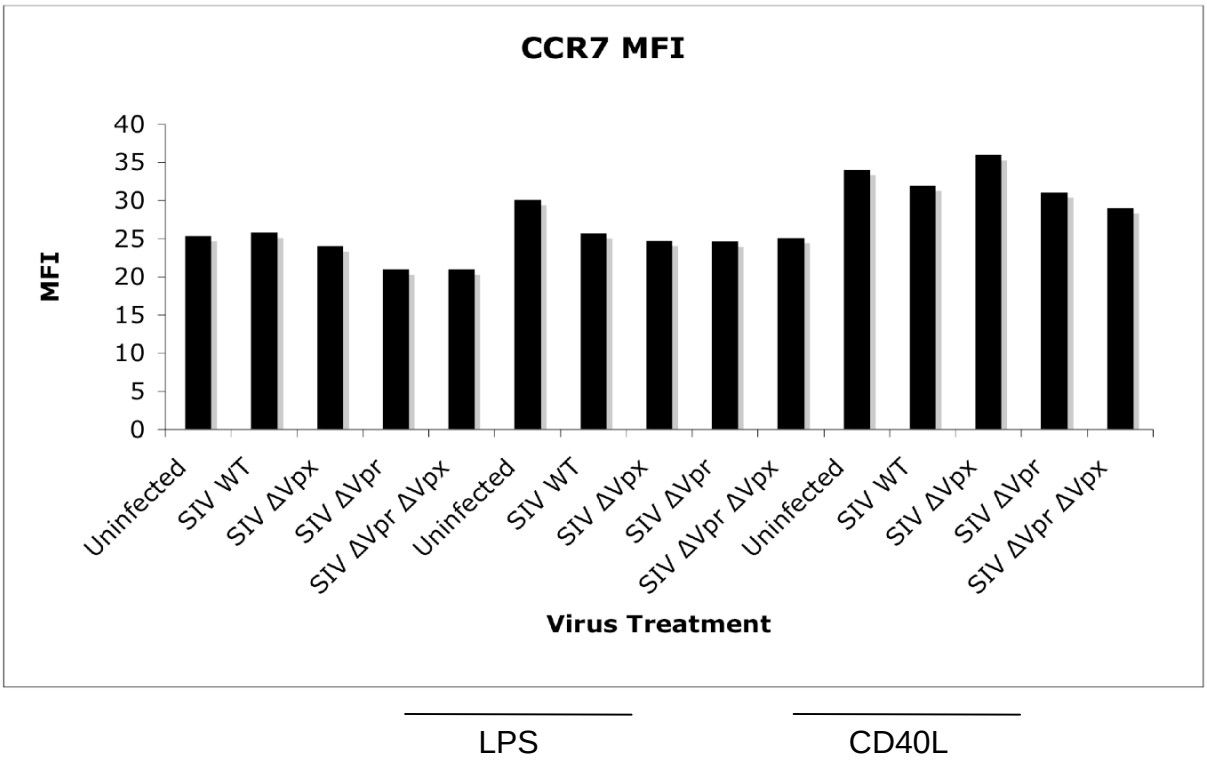
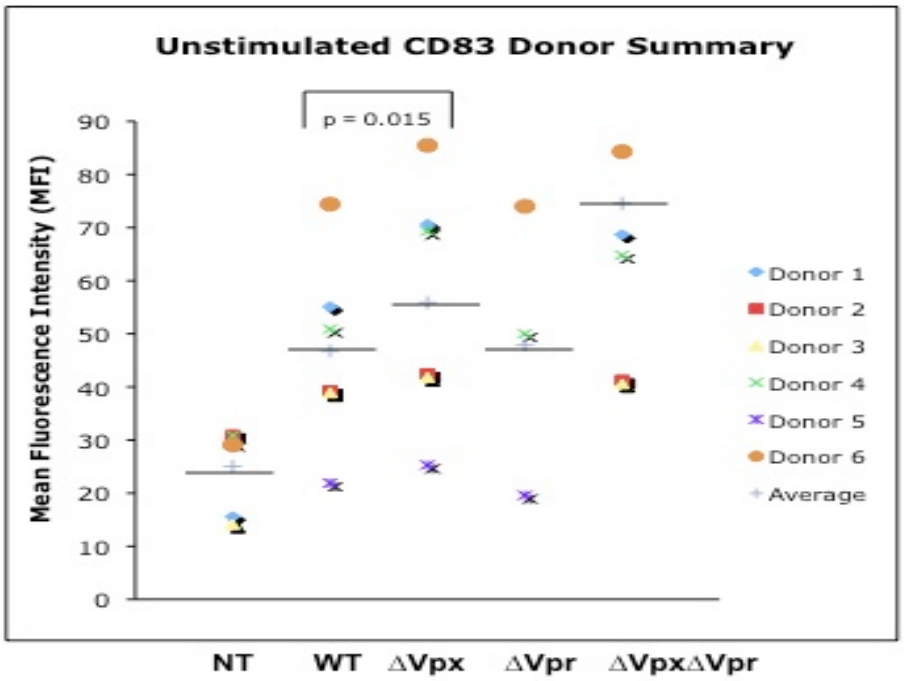


Figure 20. ***CCR7 MFI Data for One Donor***
Represents mean fluorescence intensity of CCR7 for all six donors. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Data was gathered by flow cytometry and software analysis by FLOWJO. Cells were either left unstimulated, as in the first 5 bars, or stimulated with LPS, as in the middle 5 bars, or stimulated with CD40L.

Each donor has great variability, but the general trends were very similar. When looking at the individual data for each donor (data not shown) we decided to compare the trends between the unstimulated samples and CD40L stimulated samples. The greatest differences could be seen between SIV PBj WT, and SIV PBjΔVpr or SIV PBjΔVpx (Fig 21-22). When each virus treated sample was compared with the other, the MFI showed a downregulation in the SIV PBj WT, and SIV PBjΔVpr treatments for each of the surface marker. This suggested that *vpx* played a role in the down regulation of these surface markers, as a rebound from an MFI of 650 to 850 was seen in CD86 and a rebound from an MFI of 65 to 75 in CD83 when *vpx* was deleted (Fig 20-21). This rebound was not seen when *vpx* alone was present and *vpr* was deleted. This same trend was not seen when the percent of cells expressing the markers was evaluated (Fig 14-17).



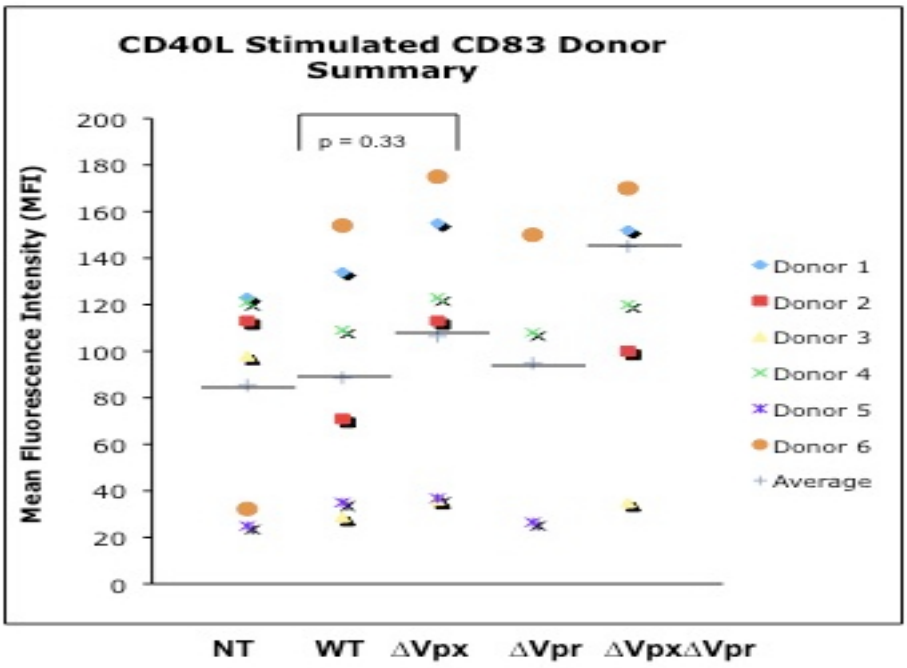
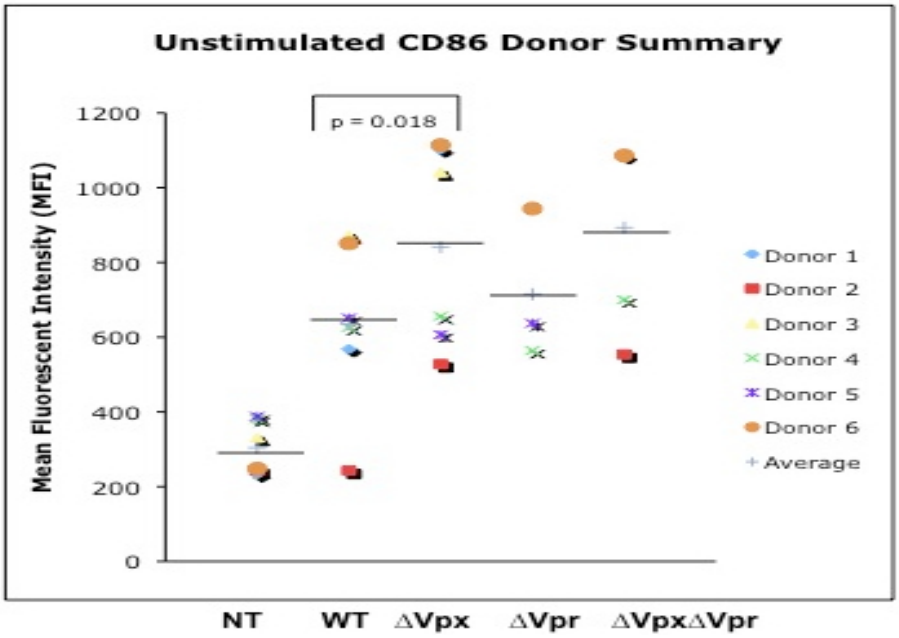


Figure 21. ***Complied Donor CD83 MFI Data***
Represents complied donor CD83 MFI information. The horizontal line in each treatment represents the mean MFI. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test.



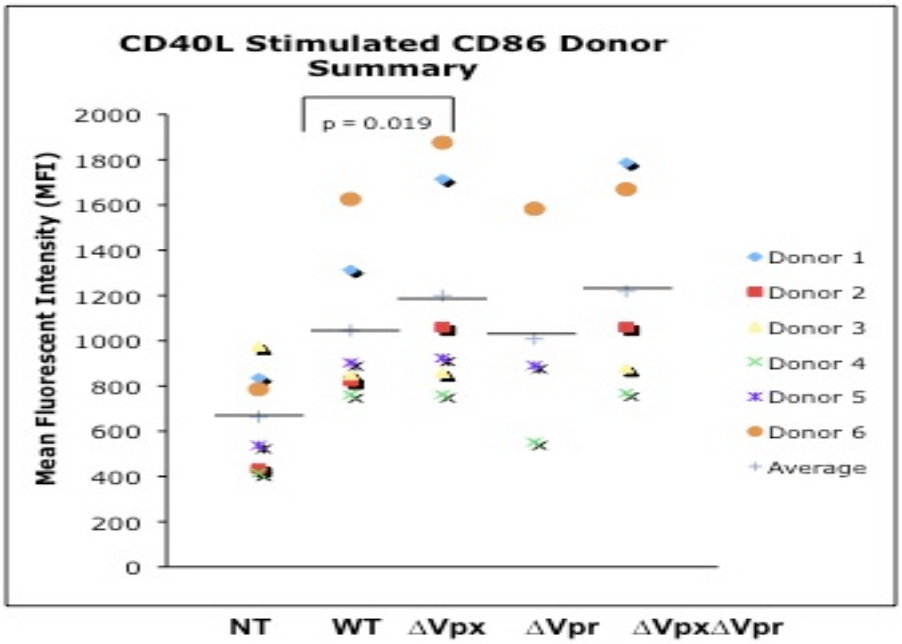


Figure 22. ***Complied Donor CD86 MFI Data***
Represents complied donor CD86 MFI information. The horizontal line in each treatment represents the mean MFI. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test.

This difference, seen in the absence of *vpx*, was also seen for the migration marker CCR7 (Fig. 22). There was a significant increase in CCR7 on the cell surface when *vpx* was deleted, while this increase was not seen when *vpr* was deleted (Fig 22).

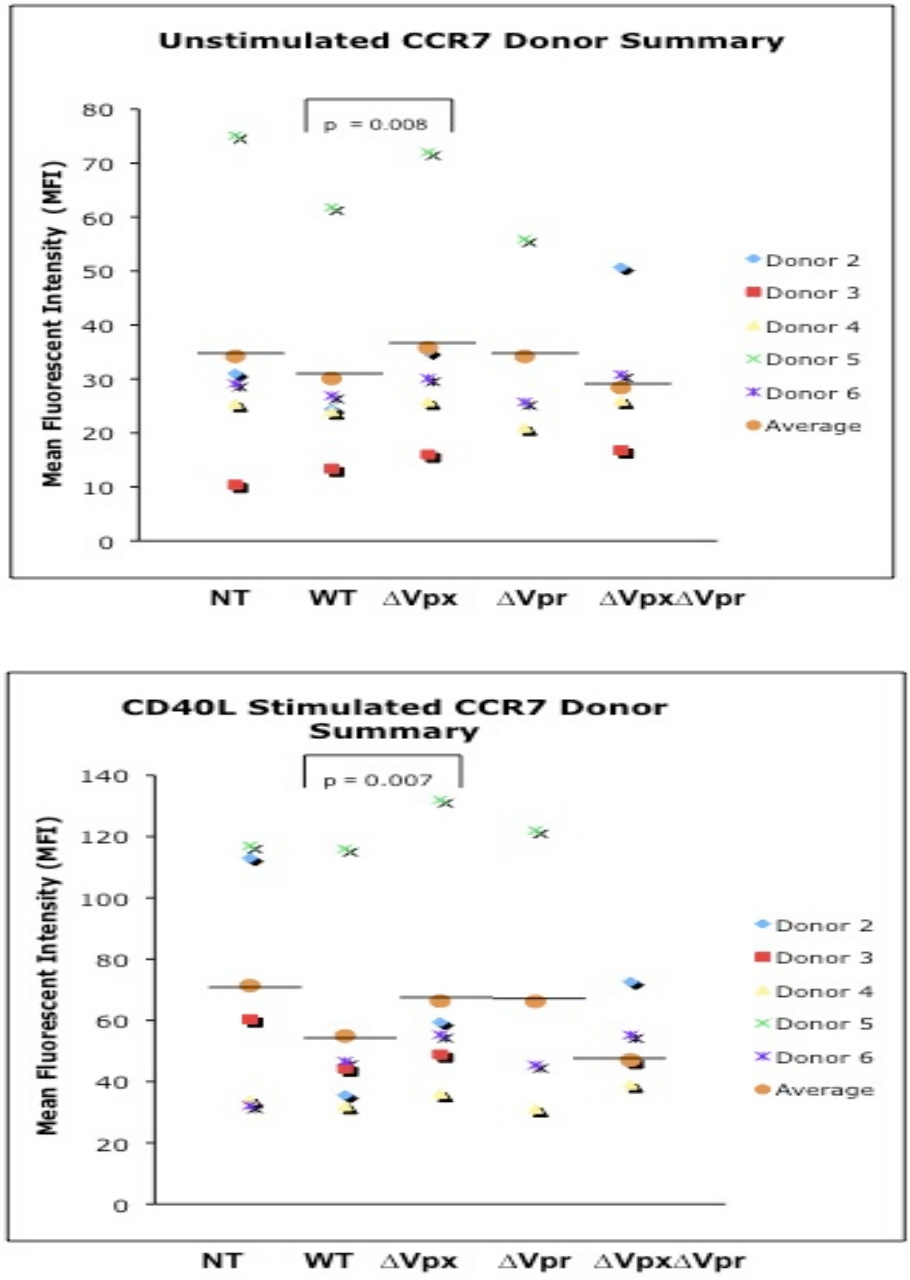


Figure 23. ***Compiled Donor CCR7 MFI Data***
Represents complied donor CCR7 MFI information. The horizontal line in each treatment represents the mean MFI. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test.

3.1.3 Determining Effect of SIV PBj Mutant Viruses on Dendritic Cell Cytokine Production

We wanted to determine if changes in certain cytokine types that would push the immune response toward type 1 and type 2 response, these cytokines are typically associated with T-cells but in this case the response will determine the ability of the DCs to activate the T-cells and push them to a type 1 or type 2 response, and we wanted to determine if SIV *vpr* or *vpx* played a role in the change. Dendritic cells were infected with each virus type and were stimulated as previously described with LPS or CD40L. The supernatants were collected after stimulation and evaluated for the presence of various cytokines. The same donors used for the surface marker data, were evaluated for TNF-α, IL-10, IL-12 p-70, and IL-15 (Fig 24-26). No IL-15 was ever detected under any treatment or stimulation (data not shown).

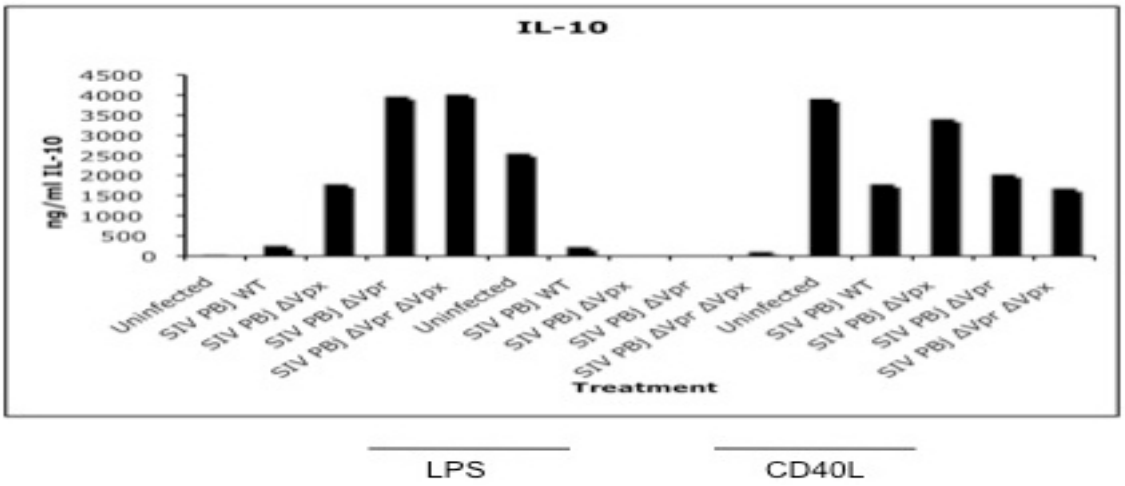


Figure 24. ***IL-10 Production in Infected Dendritic Cells***
Representative of the IL-10 levels for all 6 donors. Unexposed and virus exposed dendritic cells were removed and the supernatant was collected for use in an IL-10 ELISA to determine the levels of the cytokine after each virus and stimulation treatment. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

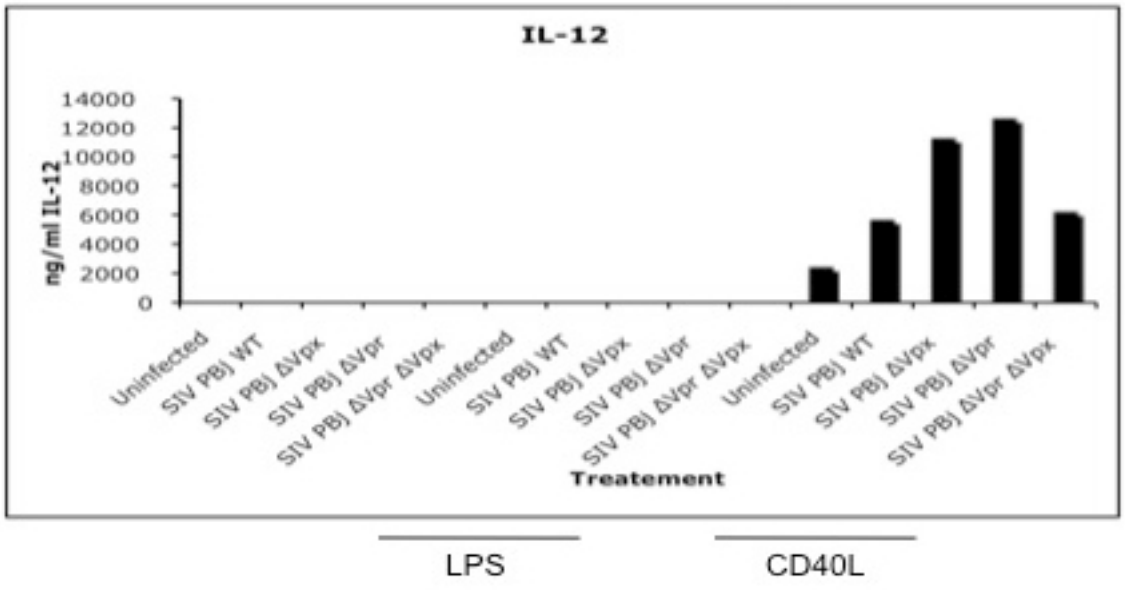


Figure 25. ***IL-12 Production in Infected Dendritic Cells***
Representative of the IL-12 levels for one donor. Unexposed and virus exposed dendritic cells were removed and the supernatant was collected for use in an IL-12 ELISA to determine the levels of the cytokine in each virus and stimulation treatment. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

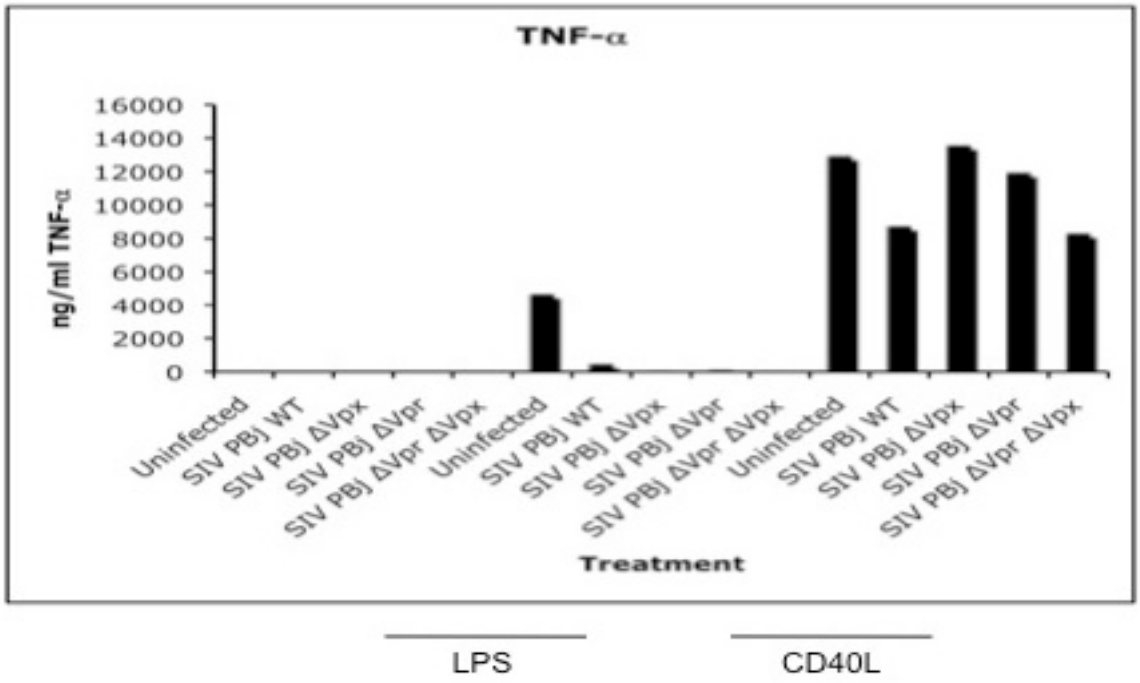


Figure 26. ***TNF-α Production in Infected Dendritic Cells***
Representative of the TNF-α levels for one donor. Unexposed and virus exposed dendritic cells were removed and the supernatant was collected for use in a TNF-α ELISA to determine the levels of the cytokine in each virus and stimulation treatment. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

Donor variation was seen in the amount of each cytokine produced, but when complied, the trend seen was similar between donors. The trend was only seen in the CD40L stimulated treatments. In the unstimulated and LPS stimulated samples, there was a very large amount of variation between donors, including some donors that did express any readable amount of the cytokines with those stimulations. We therefore complied all of the results using the CD40L stimulated samples. There was an increase in each of the cytokines, from 1000 ng/µl to 6000 ng/µl of IL-10, from 19,000 ng/µl to 30,000ng/µl of IL-12, and 20,000 ng/µl to 30,000 ng/µl of TNF-α, when *vpx* was deleted (Fig 27-29). This increase was reversed upon the deletion of *vpr* leaving only *vpx* alone for IL-10 and IL-12, but deleting *vpr* did not reverse this trend in TNF-α production (Fig 27-29). The increase in each of the cytokines upon the deletion of *vpx* was statistically significant.

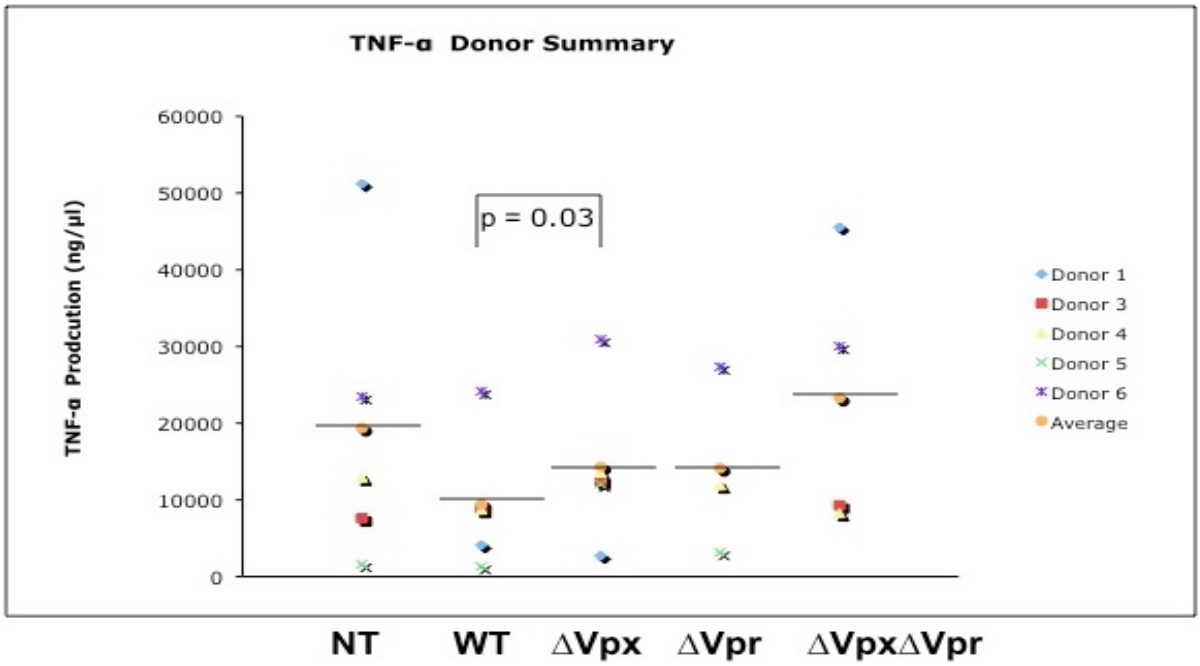


Figure 27. ***Compiled TNF-α Data for All Donors***
Represents complied donor TNF-α levels detected. The horizontal line in each treatment represents the mean cytokine level. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test. Donors 1 and 2 are missing from SIV PBjΔVpr treatment, because that virus was not available for those two donors.

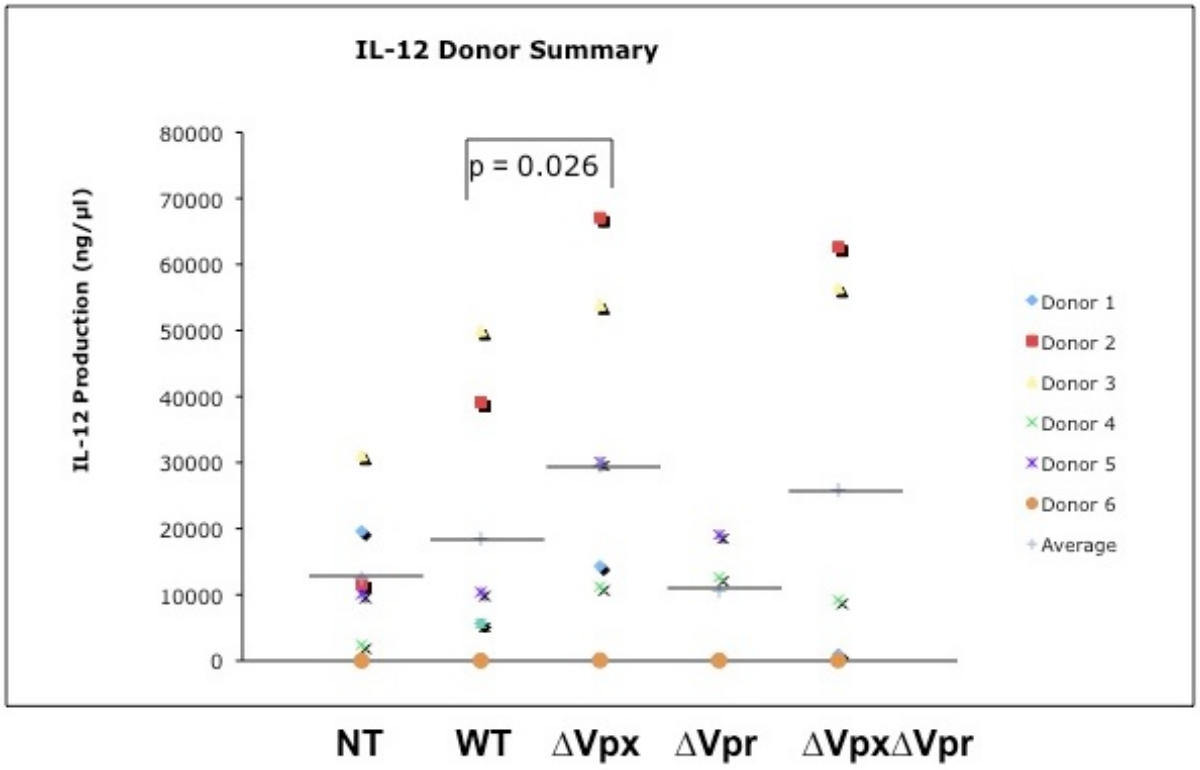


Figure 28. ***Compiled IL-12 Data for All Donors***
Represents complied donor IL-12 level detected. The horizontal line in each treatment represents the mean cytokine level. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test. Donors 1 and 2 are missing from SIV PBjΔVpr treatment, because that virus was not available for those two donors.

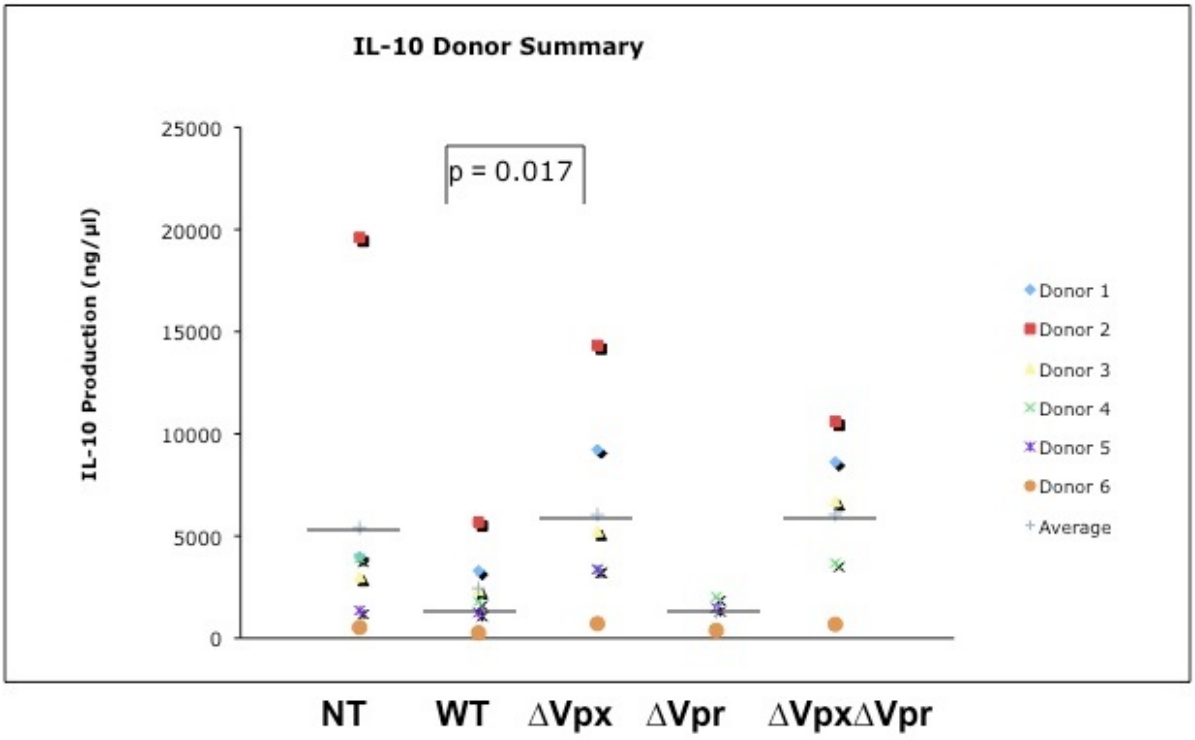


Figure 29. ***Compiled IL-10 Data for All Donors***
Represents complied donor IL-10 levels detected. The horizontal line in each treatment represents the mean cytokine level. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test. Donors 1 and 2 are missing from SIV PBjΔVpr treatment, because that virus was not available for those two donors.

3.1.4 Functional Analysis of NK Cells in the Presence of Wild type and Mutated Virus

We wanted to examine the effects of *vpr* and *vpx* on the functional capacity of NK cells during infection of bystander cells. PBMCs were collected and stimulated with IL-2 and PHA-P for 24 hours, and then infected with SIV PBj WT, SIV PBjΔVpr, SIV PBjΔVpx, or SIV PBjΔVprΔVpx virus for 4 or 6 days. Cells were collected and then screened for the presence of the degranulation marker CD107a on day 4 and day 6. This specific marker was chosen as a marker of degranulation, and when present, and shows that the cell is capable of killing a target cell and has full functional capacity. It was determined that during infection the functional capacity of NK cells is reduced and the Vpr protein plays a role in this process. CD107a levels decreased significantly in the presence of virus and this decrease was reversed significantly, by nearly 50%, when the *vpr* gene was deleted (Fig 30-32). When *vpx* was deleted, there was little change as compared to WT infection. This trend was seen in all three donors, despite donor variation seen. The trend was consistent between donors (Fig 31). This trend was also seen in the percent of cells expressing CD107a as well as the amount, measured by MFI, of CD107a expressed on the surface of those cells (Fig 32). In both cases the double mutant served to back up the results seen in the *vpr* deletion experiment, and a decrease in the expression levels was seen.

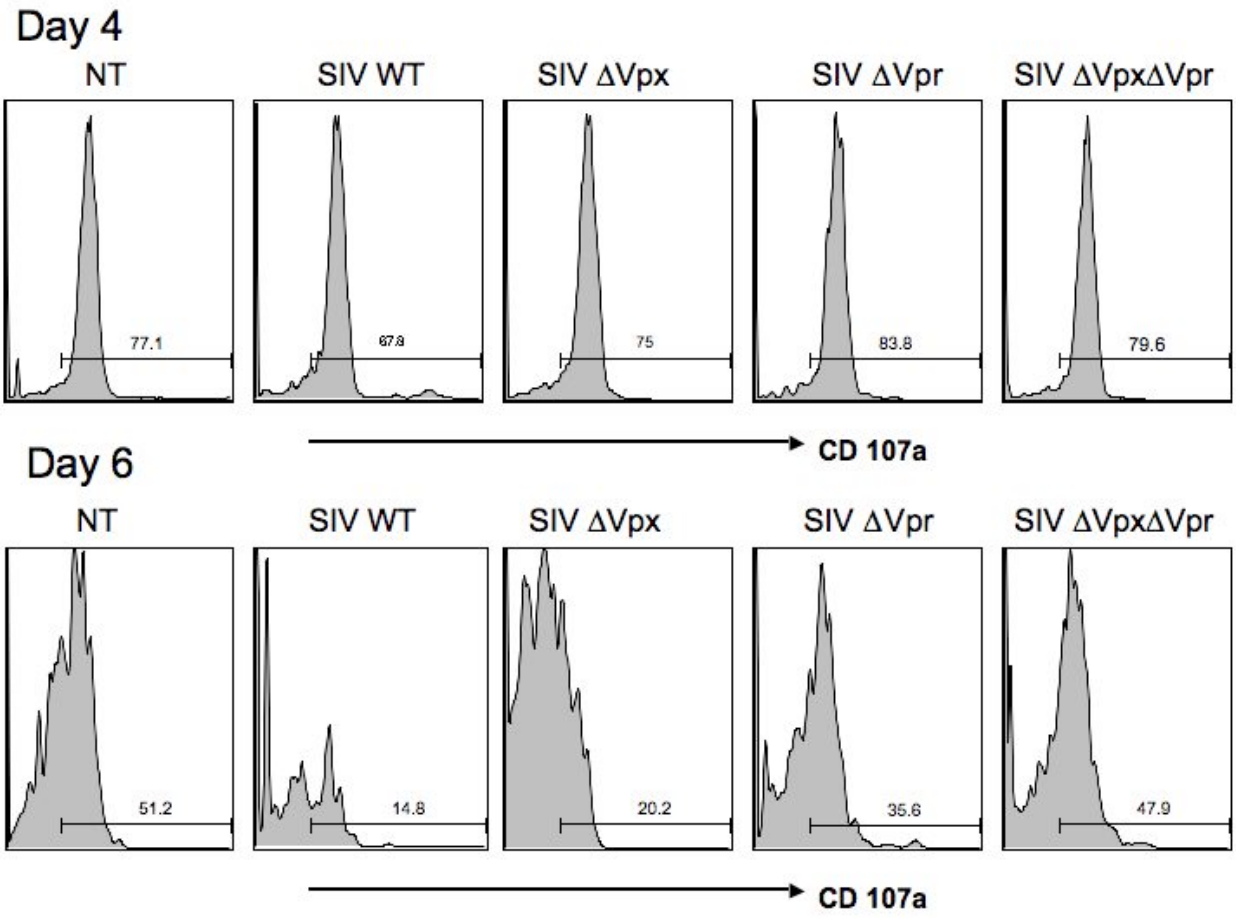


Figure 30. ***Representative Donor Analysis of CD107a Expression on NK Cells***
PBMCs were infected with 100ng of each virus for 4 or 6 days. Cells were then stimulated with K562 cells, as stated in the methods, and stained with CD107a to assess degranulation of NK cells. Cells were evaluated by flow cytometry and analyzed with FLOWJO software. Gating was on CD3-CD56+ cells, which were then evaluated for expression of CD107a. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

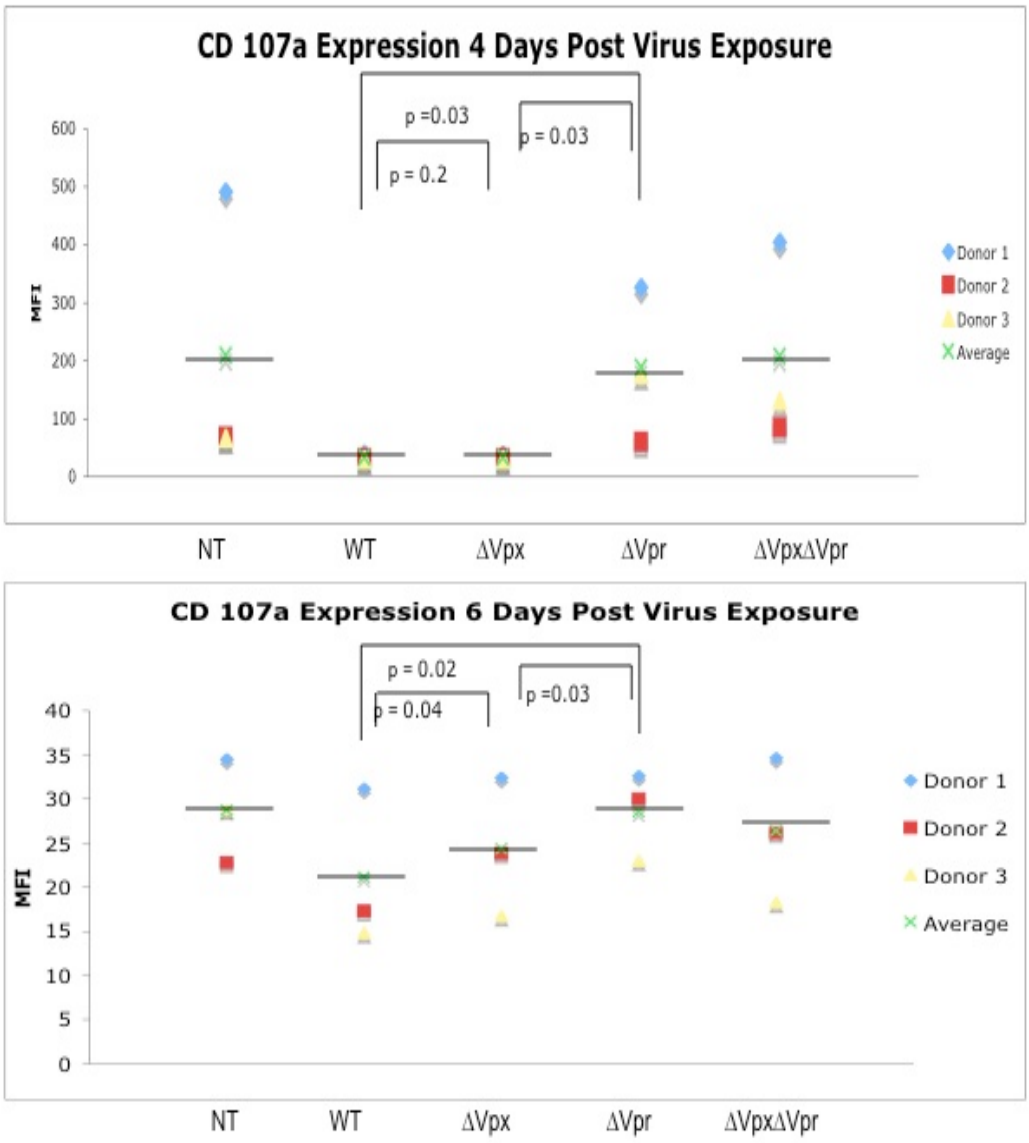


Figure 31. ***Compiled Donor Data for CD107a Expression on NK Cells as Measured by MFI***
Represents complied donor CD107a levels detected on cells. The horizontal line in each treatment represents the mean fluorescence intensity for the cell surface CD107a. Each donor is represented by a different colored symbol. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Significance was determined by paired student t-test.

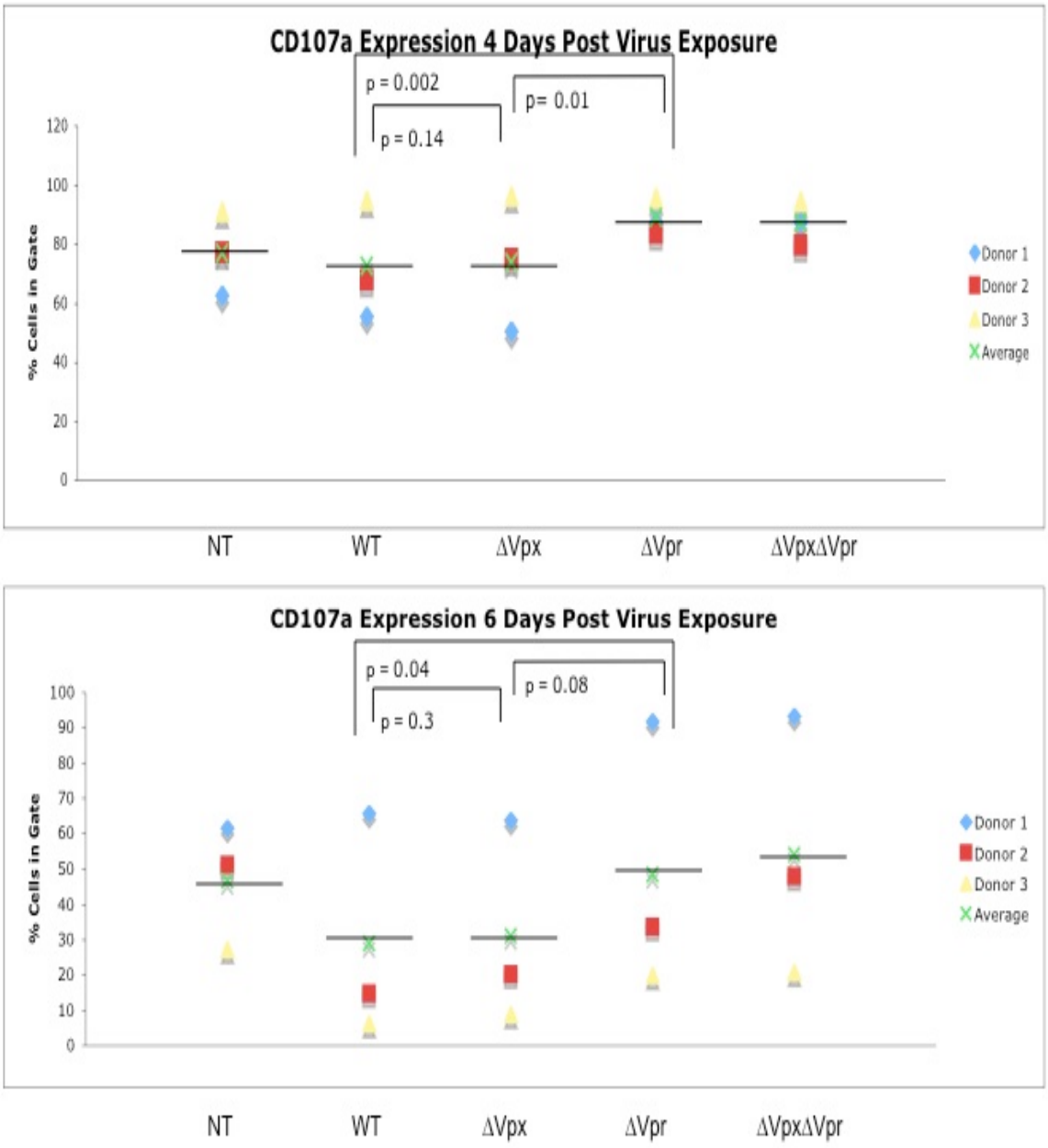


Figure 32. ***Compiled Donor Data for Percent Positive CD107a Cells***
Represents complied donor CD107a levels detected on cell surface. The horizontal line in each treatment represents the percent positive cell number for the surface marker on day 4 and day 6. Each donor is symbolized by a different colored symbol. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr. Significance was determined by paired student t-test.

3.1.5 NK Subset Distribution Changes in the Presence of Wild type and Mutated Virus

Next, we wanted to determine the effects of *vpr* and *vpx* on population changes, a shift in the functional versus anergic populations, within the NK cells as a result of infection among bystander cells. Cells were screened on day 4 and day 6. Cells were evaluated by flow cytometry for CD16+ cells within the CD3- and CD56+ populations. Results suggested that viral infection changes the subset population of NK cells in the presence of virus, either wild type or mutants. During infection there was an increase in the CD16+, or anergic, cell population and a decrease in the number of CD16-, functional NK, cell subset [67]. Infection with the Vpr mutant virus showed a significant decrease, by nearly 42%, in the CD16+ population as compared to the Vpx mutant or wild type virus (Fig 33-35). This change was seen at both day 4 and day 6. This was also consistent between donors, despite variation in the amount of change. Both the percent of CD16+ cells as well as the amount of CD16+, MFI, present, showed significant changes in the presence of the wild type and mutant viruses (Fig. 34).

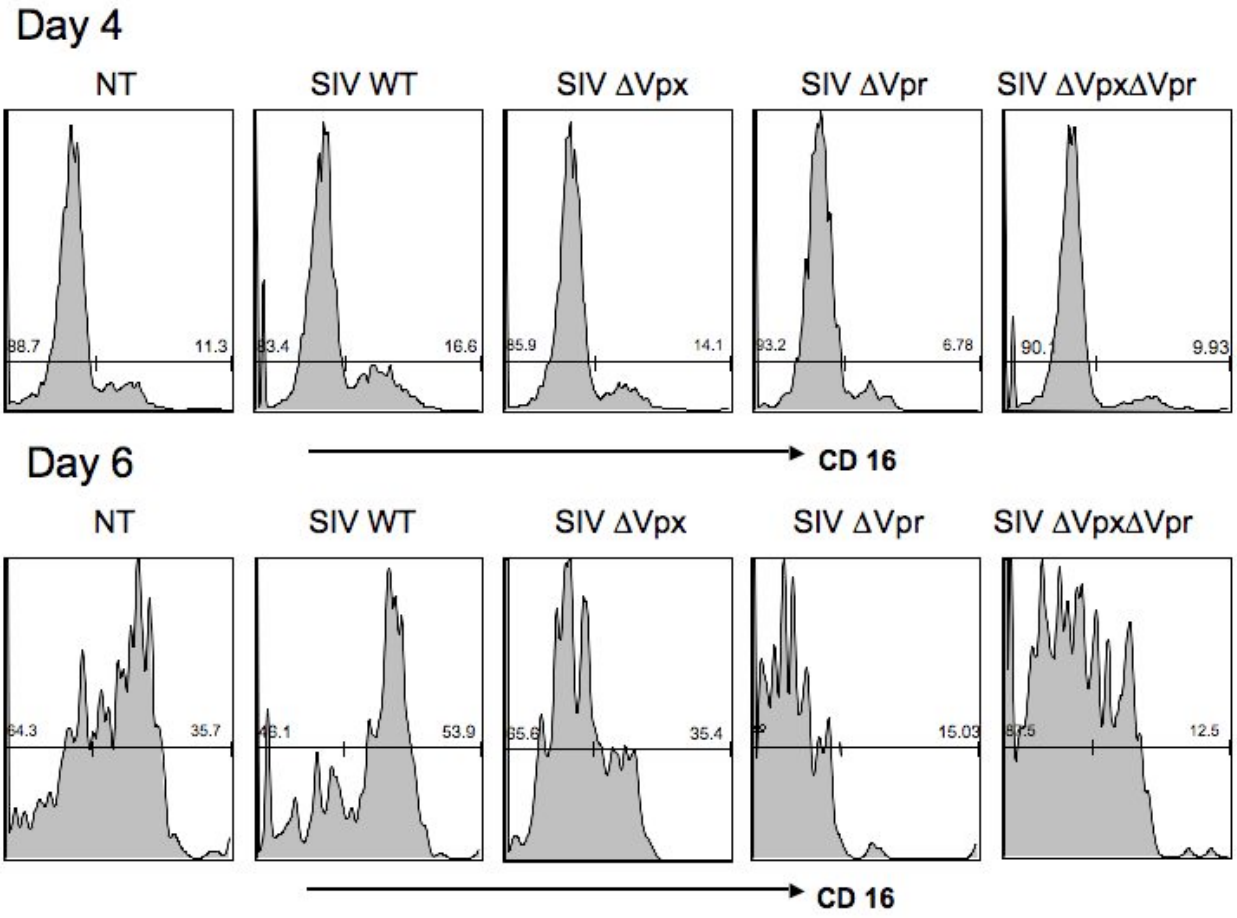


Figure 33. ***Representative Donor for CD16 Expression on NK Cells***
PBMCs were infected with 100ng of each virus for 4 or 6 days. Cells were then stimulated with K562 cells, as stated in the methods, and stained with CD3, CD56, and CD16. Cells were evaluated by flow cytometry and analyzed with FLOWJO software. Gating was on CD3- CD56+ cells, which were then evaluated for expression of CD16. The number on the right represents the percent CD16+ and the number on the left represents the percent CD16-. Donors here were the same donors used in the previous CD107a experiment. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

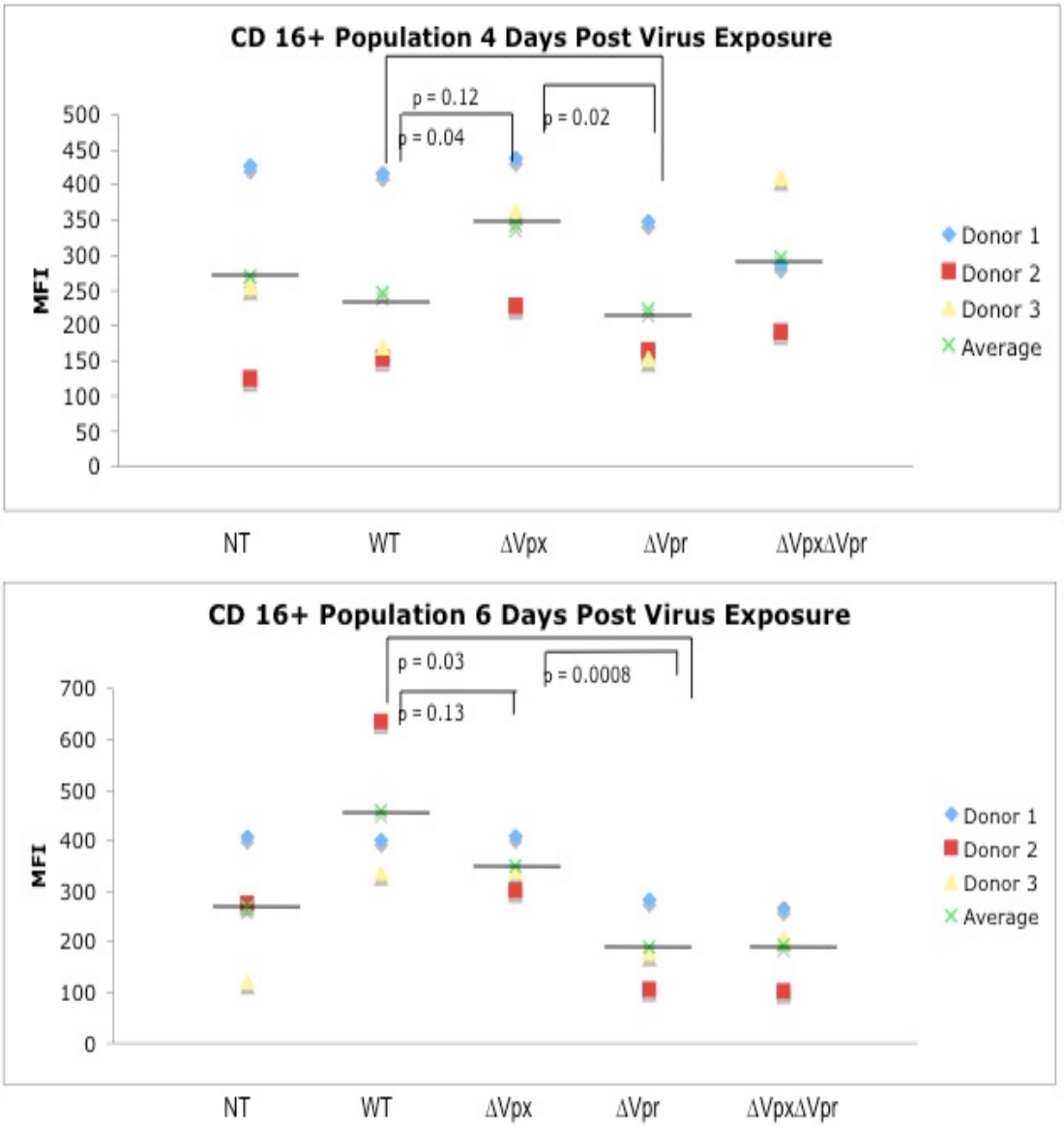


Figure 34. ***Compiled Donor Data for CD16 Expression on NK Cells and MFI***
Represents complied donor CD16 levels detected. The horizontal line in each treatment represents the mean MFI for the surface marker. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test. Gating was on CD3- CD56+ cells, which were then evaluated for expression of CD16. Donors here were the same donors used in the previous CD107a experiment. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

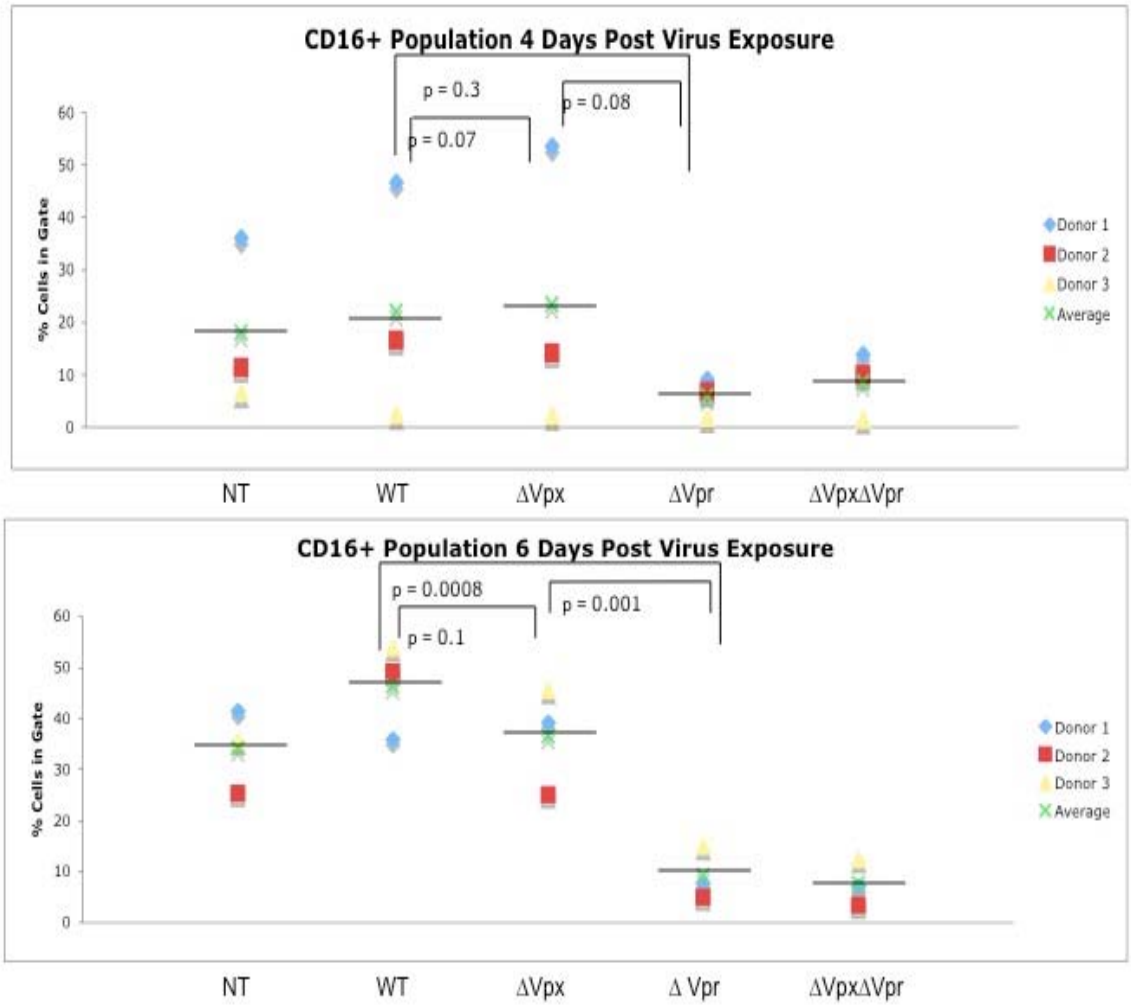


Figure 35. ***Compiled Donor Data for Percent Positive CD16 NK Cells***
Represents complied donor CD16 levels detected. The horizontal line in each treatment represents the mean fluorescence intensity for the surface marker. Each donor is symbolized by a different colored symbol. Significance was determined by paired student t-test. Each donor was left either unexposed or exposed to WT, SIV PBjΔVpx, SIV PBjΔVpr, or SIV PBjΔVpxΔVpr.

4.0 DISCUSSION

4.1 THE EFFECTS OF SIV VPR AND VPX ON DC MATURATION AND CYTOKINE PRODUCTION

It has become increasingly apparent that controlling the virus initially upon infection by priming cells from both the adaptive and innate immune systems and clearing the body of virally infected cells before latent cells develop is a key part of an effective immune response. The viral set point, or the steadiest amount of detectable viremia following the acute phase of infection, has been shown to correlate with that rate of disease development and death of an infected individual [68]. The virus, unfortunately, does everything in its power to ensure that it has the upper hand. Dendritic cells, one of the first responders, are rarely productively infected, but play a very large role in both the immune response as well as viral trafficking [69]. HIV-1 uses its accessory proteins to alter the functional capacity of these cells and their ability to stimulate other immune cells to mount a response to the infection [65]. By understanding the mechanisms through which this occurs, we may be better able to treat the infection early on. We wanted to ensure that the same dysregulatory functions seen in HIV-1 are also seen with SIV. We also wanted to determine if the dysregulatory functions specifically controlled by HIV-1 *vpr* are also controlled in SIV by *vpr* or *vpx*.

My studies indicate that many of these mechanisms for altering maturation capacity and migration of DCs are also present during SIV infection. In HIV-1 infection, *vpr* has a role in controlling many of these changes, and our results indicate that in the case of DC immune dysfunction, SIV *vpx* plays the predominant role in controlling this process [8]. Similar to HIV-1 Vpr, SIV Vpx is packaged in the virion [8]. Because most DCs are not directly infected, but are instead exposed to virus and viral particles by uptake of the virus, without subsequent productive infection, it seems that *vpx* would be the most likely cause. This is because *vpx* is present in the virion and does not need to be produced *de novo* to cause an effect. In my experiments we were able to show that nearly all DCs take up virus. The more virus in the environment, the more DCs are able to take up virus, but even at low concentrations of virus, a large number of DCs are able to take up to virus. This suggests that a large number DCs would be exposed to the structural proteins of the virus, as well as any proteins packaged within the virion, including vpx. SIV Vpr would not be made until productive infection had been established and Vpr could be made de novo, which helps explain the lack of SIV Vpr involvement in this dysregulation.

During the course of our study it became very apparent that SIV *vpx* played a large role in the dysregulation of DCs. It played a role in both inhibiting the maturation of the cells as well as decreasing the expression of migration markers on the surface of the cells. In our studies we looked at CD83 and CD86 expression in the presence of WT as well as mutant viruses, defective for *vpr* and/or *vpx*. This downregulation would decrease the maturation capacity of the cells preventing them from being better able to activate T-cells, allowing them to begin fighting the infection. Through these experiments we were able to determine that SIV Vpx plays a role in down regulating both of these surface markers. When Vpx was deleted, the effects were

diminished and a rebound in the surface marker was seen. The amount of these markers expressed on the surface was decreased, but the number of cells expressing the markers did not decrease, and remained relatively the same.

This same trend was witnessed with the migration marker CCR7. When this marker is expressed the cell is capable of migrating to lymph nodes [70]. This would allow the cells to present antigen to other immune cells. SIV Vpx decreased the amount of CCR7 present on the surface of exposed DCs. This downregulation has the possibility of preventing cells from being able to "warn" other immune cells and begin mounting a response. When *vpx* was deleted this response was abrogated and there was a significant increase in the amount of CCR7 on the surface of the cells. Similar to the effects seen on CD86 and CD83, the number cells expressing CCR7 did not change, but the amount of it present on the cells was the biggest indicator of change.

Along with the changes seen in the ability of these DCs to mature and migrate, HIV-1 Vpr has also been shown to cause changes in the cytokines these exposed cells produce. Exposed cells are more likely to produce Th2 cytokines, like IL-10 and less likely to secrete Th1 cytokines, such as IL-12 and TNF-α. Altering the cytokine environment also prevents the recruitment of cells, such as NK cells, cytotoxic T cells, macrophages and others, that would be more likely to kill infected cells, preventing reinfection of other cells. These cytokines activate transcription in different cell types allowing them to upregulate replication of different cell types in response to the cytokines. Each cytokine group elicits a different response and allows different cell types to replicate and mature in response to an infection. This allows the virus to further establish

infection by recruiting cells it can infect and prevent cells that could fight off infection from coming into the area.

During the course of our study we determined that SIV Vpx behaves similarly to HIV-1 Vpr in its ability to alter the cytokines secreted by exposed cells. We measured IL-12, IL-10, TNF-α, and IL-15. Similar changes were seen with IL-12, IL-10, and TNF-α. In the presence of Vpx, each cytokine was decreased, but when *vpx* was deleted there was a rebound in the amount of cytokine secreted. Based on previous studies done with HIV-1 Vpr, this response was expected for both IL-12 and TNF-α, but was not expected for IL-10 [41]. In previous studies, IL-10 was increased in the presence of HIV-1 Vpr [41]. The reason for this difference cannot be explained by the results alone, and little literature exists on these cytokines in relation to SIV, so more work would need to be done to determine the cause for this discrepancy. Soluble IL-15 was not able to be detected in any of these samples and was therefore not included.

DCs play a large role in the initial stages of infection and HIV-1 has developed many ways of insuring these pivotal cells are reduced in their ability to function in the ways that would best allow them to help establish an effective immune response. It is important to learn how the virus manipulates the cellular environment and to take this information and convert it into a treatment strategy. The development of an animal model is key in this process, and showing that despite having a slightly different genomic set up, with two genes, vpr and vpx, where HIV-1 has only one, vpr, we see that SIV is able to function in a similar manner, with similar dysregulation of immune function due to these proteins. This will be an important step in that direction.

4.2 THE EFFECTS OF SIV VPR AND VPX ON NK CELL FUNCTION

The involvement of the innate immune system in protection from infection has come into focus more in recent years. Because HIV-1 specifically targets T-cells and cells of the adaptive immune system, it has been the predominant theory that enhancing the adaptive immune response would be the key to beating the virus in the long run. In other words, the longer one's T-cells and adaptive immune cells can fight, the greater one's likelihood for resistance to development of disease. While this is true, prevention of infection and keeping viral levels down in the initial stages, hours and days, of infection is mainly determined by the innate immune system [70]. It has also been discovered that HIV may not infect or target innate immune cells, but the presence of virus and the infection of other cells within the body does pay a significant toll on the bystander innate immune cells. These effects then make it harder for innate immune cells to help in the fight against infection both against HIV as well as against opportunistic infections that develop as result of the loss of adaptive immune cells. HIV-1 Vpr has been shown to have specific roles in this dysregulation of innate cell function, in some cases, by decreasing the functional capacity of the innate cells, such as NK cells [70]. In my study we wanted to determine if these same effects are seen in SIV as well, and to determine if the SIV *vpr* or *vpx* genes had any part in this dysregulation.

Our study shows that these same effects are seen in SIV infection as well as HIV-1, and that the SIV accessory gene *vpr* but not *vpx* has a role in this dysregulation. As previous studies have shown, SIV Vpx, is packaged within the virion, similar to HIV-1 Vpr [8]. This allows cells to be exposed to the protein before *de novo* synthesis of viral proteins takes place. Proteins present within the virion would likely cause effects at early time points during infection. This protein is

likely to affect cells that are directly targeted by the virus, or those cells that function as APCs and have direct contact with the virus. During the initial acute infection, when viral titers are high, bystander cells may be affected, but during times when viral titers are low, bystander cells are less likely to be affected by virion associated proteins. HIV-1 or SIV does not infect NK cells, but the T-cells and macrophages present in the PBMC milieu are likely to be productively infected [70]. This infection will produce a greater amount of virus as well as cell death increasing the concentration of viral proteins in the environment [65]. The presence of these viral products within the environment has the potential to then affect bystander cells that are not infected by the virus. This phenomenon gives some insight into the ability of SIV Vpr to have a role in the dysregulation of these cells, while not being productively infected by these cells.

During the course of our study it became apparent that NK cells are indeed affected by SIV infection. We were not able to distinguish the cells that were infected within these samples, but it is assumed based on previous studies that the T-cells and macrophages within these samples are the source of the productive infection. Studies were done using p27 antibody staining in PBMCs to show that productive infection does occur, but the specific cell types infected were not identified (Fig 7). It also became apparent that SIV *vpr* played a significant role during infection in the dysregulation of NK cells, while *vpx* did not. After 4 days of infection CD107a surface levels, after stimulation with K562 cells, were decreased with SIV WT infection. The decrease was similar when *vpx* was deleted and only SIV *vpr* was present. This down-regulation was completely eliminated when *vpr* was deleted and a rebound to near that of the untreated sample was seen. This suggests that *vpr* plays a greater role than *vpx* in this down-regulation.

We then assessed the subset distribution of NK cells. There are functional and non-functional subsets of NK cells. Those cells that are CD3-, CD56+ and CD16- are the functional subset [67]. Those cells that are CD3-, CD56+ and CD16+ are NK cells that have been pushed into anergy and are no longer functional cells [67]. They are not dead and have not yet gone through apoptosis, but they no longer are able to perform any of their killing functions, or any other function. It is possible that this change in subsets is why these cells are not producing the degranulation markers. We looked at the subsets in the presence of virus after 4 and 6 days. Cells were stimulated with K562 cells and were then stained for CD3, CD56, and CD16. It became apparent that even after 4 days, there was indeed a shift in the subset distribution of the NK cells. During SIV WT infection there was a shift to a CD16+, or anergic, population. This suggests that productive infection does play a role in the dysregulation of NK cells. When SIV *vpx* was deleted, there was little change in the percent of cells expressing the marker or the amount of CD16 on the cell surface, or MFI. When *vpr* was deleted, however, there was a significant decrease in the percent CD16 positive and MFI of the marker on the cell surface. This suggests that SIV *vpr* does indeed play a role, not only in dysregulating the functional capacity of NK cells, but also pushes them into anergy, rendering them useless and completely non-functional.

As previously stated, NK cells are not infected by HIV-1 or SIV. This means that the infection of other cells in the PMBC milieu is the main avenue for the dysregulation of these particular cells. The fact that SIV Vpr is also the protein that is causing the functional and subset changes supports this argument. SIV Vpr is only present during infection, and would only be able to cause these effects if infected cells were making it during viral replication and transcription.

Despite the fact that Vpx is required for infection of non-dividing cells like macrophages, and that it is also known to help with the transport of viral DNA to the nucleus, infection in T-cells and other bystander cells still occurs and deleting this gene still allows for the dysregulation of NK cells with little change from that of SIV WT infection.

SIV infection can be used as a model of HIV-1 infection, and despite a different genomic composition, the proteins produced have analogous functions. The HIV-1 *vpr* gene has been shown to have specific roles in the dysregulation of NK cells and the SIV *vpr* and *vpx* genes, specifically SIV vpr in this case, behave in a similar manner. This information can be used to do more studies to determine the specific mechanism of this interaction, and to then find ways of either eliminating this interaction, or decreasing it to allow these immune cells to eliminate the virus early and prevent reinfection and disease.

4.3 PROPOSED EFFECTS OF SIV VPR AND VPX ON INNATE AND ADAPATIVE IMMUNITY

The specific actions of these proteins remains to be studied, but we have determined that the SIV Vpr and Vpx proteins do behave in a similar manner to that of HIV-1 Vpr. These two proteins split the functions of the one HIV-1 protein, but same end function is seen, dysregulation of the immune system. This dysregulation is seen in both arms of the immune system, adaptive and innate. The virus infects macrophages in the periphery and is picked up by DCs. These DCs are susceptible to the virion-associated proteins carried by the virus. The cells take the virus particles to the lymph nodes, and pass them to susceptible T-cells, which subsequently become infected with the virus. The T-cells produce virus, which is then picked up by more DCs. The virion-associated proteins, such as Vpx, cause a downregulation of surface maturation markers and migration markers (Fig 36). This down regulation makes it harder for these cells to appropriately activate naive T-cells and B-cells to combat the growing infection. While this is happening, the infected T-cells are also dying and releasing viral proteins into the cellular environment. This release of proteins affects other cells that may not be infected within the environment, including NK cells. These cells are affected by SIV Vpr protein, which causes many cells to loose degranulation markers from the surface, and to also be pushed into an anergic state (Fig 36). This decreases the number of functioning NK cells, decreasing the number of cells capable of fighting off the growing infection even further. The combination of these two events, as a result of the affect of these two proteins appears to play possibly significant role in the ability of the virus to gain the upper hand during the course of the infection.

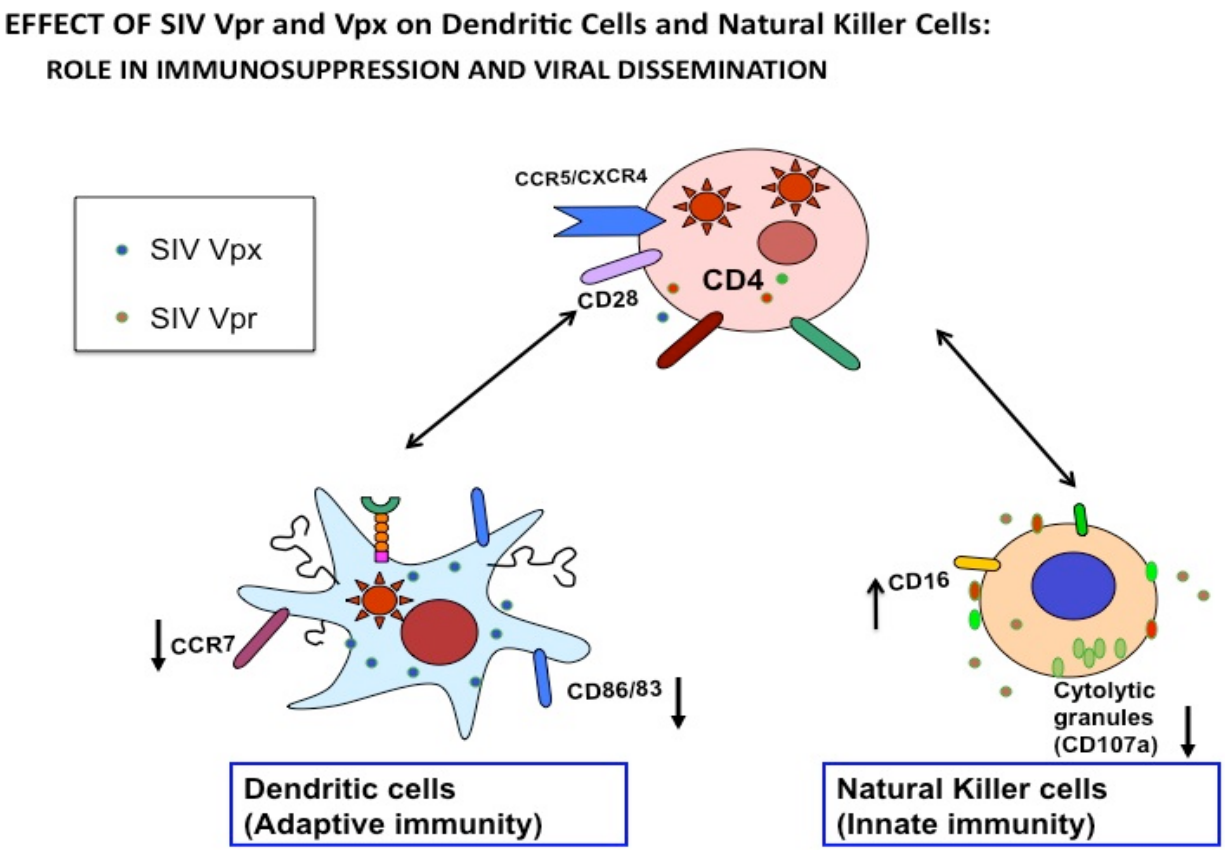


Figure 36. ***Combined Effect of SIV Vpr and Vpx on Innate and Adaptive Immunity***
This is a schematic representation of the affect of SIV Vpr and Vpx on the two arms of the immune system. The red sun shaped objects are the SIV virus particles, the blue dots are Vpx protein particles, and the brown dots are Vpr protein particles.

This study has shown a number of similarities between HIV-1 Vpr and SIV Vpr and Vpx. These proteins, in conjunction with other viral proteins, dysregulate the immune system so effectively, that they give the virus the upper hand in may instances. If we could target these proteins specifically early on during infection, we may be able to stop the infection of non-dividing cells such as macrophages, which can serve as a reservoir for the virus. We may also be able to prevent the dysregulation of important immune cells such as NK cells, which have proven to be crucial in fighting off HIV infection, as well as opportunistic infections during AIDS. If these proteins can be targeted, and rendered inactive or incapable of completing their dysregulatory functions, we may finally be able to turn the tables on this pandemic disease.

5.0 FUTURE DIRECTIONS

The findings we have presented here have offered a key glimpse into the interplay between SIV and DCs. These results offer a starting point to further experimentation in the area. It will be necessary to conduct further experiments to verify these results, as well as expand upon them.

One of the biggest obstacles has been the lack of reagents for use in SIV research. We were not able to verify that the viruses expressed the correct proteins and didn't express proteins they were intended not to. This would best be done through western blotting, but the appropriate antibodies for these proteins were not accessible.

RT PCR analysis could be done to verify much of the data collected on the expression of surface markers. This same technique could be used to determine if the changes in the cytokine expression are a result of decreased RNA or if it is simply the amount protein released. It is also necessary to determine why this disconnect exists in the expression of IL-10 in the presence of SIV.

The NK data gives some insight into the relationship between SIV *vpr* and the dysregulation of NK cells during SIV infection. Other experiments need to be done to ensure that this

dysregulation is truly a result of infection such as looking for evidence of infection by perhaps testing for p27 antibodies within the supernatant at each time point.

Along with testing for changes in subset populations, apoptosis should also be looked at. HIV-1 *vpr* has been known to induce apoptosis in cells and it is possible that this is also occurring in the NK cell population as well as the shift to anergy.

Tests can also be done to verify the decrease in the CD107a expression or the functional capacity of these cells such as a chromium release assay. LDH assays could also be used but during our experiment this assay was used and the results were not consistent or reliable. Chromium release may be a better choice to test the functional capacity of these cells.

Other groups have also looked at cytokine expression and receptor expression when looking at NK cells and their functional capacity during infection. It would be good to look at the effects of virus on these aspects as well, to determine if SIV *vpr* has roles in other areas of NK dysregulation, or if other proteins cause these aspects.

We have shown that SIV does cause changes in the NK cell population, but these many not be the only changes and these changes must be confirmed by other techniques.

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