LETHAL HYPERMUTATION INDUCED BY THE INNATE CELLULAR RESTRICTION FACTOR APOBEC DESTROYS HIV-1 IN INFECTED HUMANIZED MICE

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DEDICATION

To Nicole, thank you for all of your love and support.

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by

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DISSERTATION

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The University of Texas Southwestern Medical Center at Dallas, 2009

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Twenty eight years after the initial case reports of what would become known as AIDS, HIV-1 remains a major health issue both globally and locally. Current antiretroviral interventions are effective at suppressing virus replication; however, they must be maintained for life as the removal of these drugs resulting in the rapid return of viremia. Novel

therapeutic approaches targeting HIV may be required to ultimately achieve a drug-free remission for infected individuals. Identification of the innate immune factor, APOBEC, has revealed one such approach.

The cytidine deaminases APOBEC3G and 3F have potent antiretroviral activity; however, they are neutralized by the HIV-1 Vif protein. *In vitro*, the absence of Vif allows the enzymatic activity of APOBEC3G and 3F to induce hypermutation of the HIV-1 genome. These observations make the Vif/APOBEC axis a tantalizing therapeutic target. The ability of APOBEC to restrict HIV *in vivo* however remains to be addressed. The adaptive capability of HIV allows the virus to develop escape mutations to evade antiretroviral drugs and persist in infected individuals; thus the possibility that HIV will be able to evade APOBEC restriction *in vivo* exists.

Humanized mice used as an in vivo model to study the capacity of APOBEC to restrict Vif-defective HIV revealed that the ability of the virus to replicate is severely crippled. HIV lacking Vif is heavily hypermutated by APOBEC, however, if the virus can restore Vif, the result is a fully replication competent virus that is resistant to APOBEC restriction.

The goal of this dissertation project was to utilize a humanized mouse model to assess the ability of Vif-defective HIV to replicate, persist and ultimately escape restriction by APOBEC *in vivo*. My conclusions are

that in the absence of Vif, restriction by APOBEC is absolute; however, this extreme selective pressure placed on the virus in some instances leads to the restoration of Vif, resulting in a fully APOBEC resistant pathogenic HIV. Thus, the Vif/APOBEC axis is an excellent candidate for antiretroviral intervention and furthermore, the humanized mouse will serve as a good model for assessing the *in vivo* efficacy of novel Vif-targeting compounds.

Acknowledgments

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Prior Publications

O'Neill, E., L. S. Kuo, J.F. Krisko, D.R. Tomchick, J.V. Garcia, J.L. Foster (2006). "Dynamic evolution of the human immunodeficiency virus type 1 pathogenic factor, Nef." J Virol **80**(3): 1311-20.

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List of Abbreviations/Acronyms

AID, Activation Induced Deaminase

AIDS, Acquired Immunodeficiency Syndrome

APOBEC, Apolipoprotein B mRNA editing enzyme, catalytic polypeptide

BM, Bone marrow

BST2, Bone marrow stromal cell surface gene 2

CA, Capsid

CCR5, chemokine (C-C motif) receptor 5

CD, cluster of differentiation

CMV, cytomegalovirus

DMEM, Dulbecco's Modified Eagle Medium

EDTA, ethylenediaminetetraacetic acid

Env, Envelope

ELISA, enzyme linked immunosorbent assay

FACS, fluorescence activated cell sorting

FBS, fetal bovine serum

GAG, group specific antigen

gp 41, glycoprotein 41

gp 120, glycoprotein 120

HRP, horseradish-peroxidase

HIV, human immunodeficiency virus

HSC, hematopoietic stem cell(s)

HEK, human embryonic kidney

Ig, immunoglobulin

In, Integrase

IL, interleukin

LN, lymph nodes

LTR, long terminal repeat

MA, Matrix protein

μg, microgram

mg, milligram

μl, microliter

ml, milliliter

Nef, negative factor

NLS, nuclear localization signal

NC, Nucleocapsid

NOD/SCID, nonobese diabetic severe combined immunodeficient mice

PB, peripheral blood

PBMC, peripheral blood mononuclear cells

PBS, phosphate buffered saline

PHA, phytohemagglutin

Pol, Polymerase

ppt, polypurine tract

PIC, pre-integration complex

Pr, Protease

RBC, red blood cell(s)

Rev, regulator of expression of viral proteins

RRE, Rev response element

RT, Reverse Transcriptase

RNase H, Ribonuclease H

SCID, severe combined immune deficient

SHIV, chimeric simian-human immunodeficiency virus

SIV, simian immunodeficiency virus

TAR, trans-activating response element

Tat, Trans-activator of transcription

TCIU, tissue culture infectious units

UNG2, Uracil-DNA glycosylase 2

Vif, Viral infectivity factor

Vpr, Viral protein R

Vpu, Viral protein U

CHAPTER ONE: Introduction to HIV

HIV is the causative agent of AIDS

The initial case reports of a new infectious disease of viral origin occurred in June of 1981 with patients presenting with atypical pneumonia caused by *Pneumocystis carinii*, oral candidal mucosal infections, and previous or current cytomegalovirus (CMV) infections (1981). Later reports from the CDC included a rare form a cancer, Kaposi's sarcoma (1982). These opportunistic infections suggested an immune suppression which in 1982 the CDC named Acquired Immunodeficiency Syndrome (AIDS). The following year, a retrovirus isolated from cultured T lymphocytes from a lymph node of an AIDS patient was identified (Barre-Sinoussi, Chermann et al. 1983) In 1986, this new virus was officially named the human immunodeficiency virus (HIV) (Coffin, Haase et al. 1986).

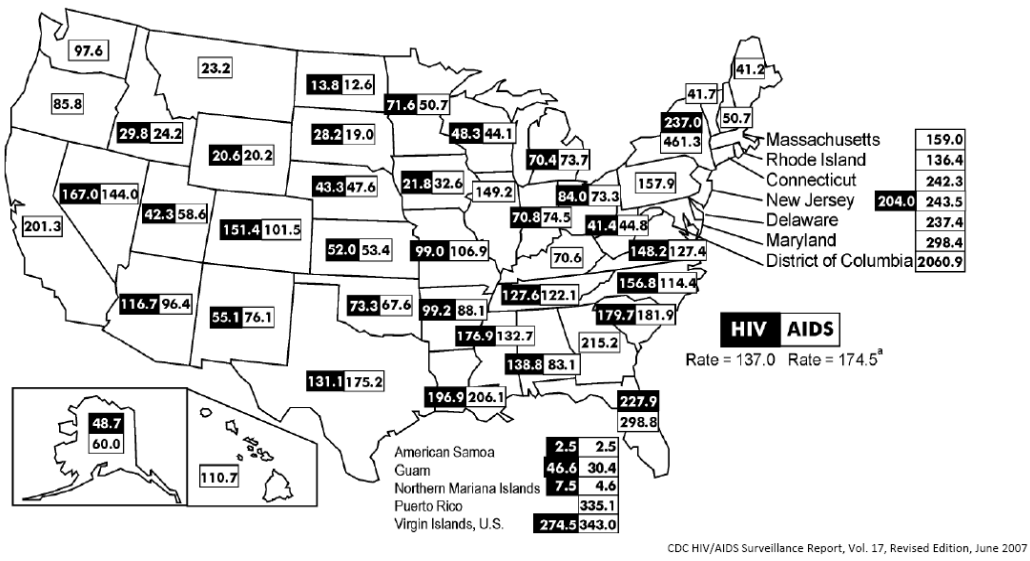

Figure 1.1 HIV and AIDS in the United States.

Estimated rates (per 100,000) for adults and adolescents living with HIV infection (not AIDS) or AIDS, 2005-United States and dependent areas (Centers for Disease Control - Revised June 2007)

Since the initial reports in 1981, the number of people infected with HIV worldwide has grown to an estimated 33 million with 2.7 million new infections occurring in 2007 (2007). In the United States alone there are approximately 1.1 million people living with HIV in 2007 (CDC 2005 revised 2007). While infection cases in the United States are widely distributed in all 50 states, the number of infected individuals in Washington DC is disproportionately high (Figure 1.1). As of 2008, 3% of DC residents were infected with HIV (2009).

The structural and enzymatic proteins: Gag, Pol, and Env

As with all lentiviruses, the HIV genome contains *gag*, *pol*, and *env* genes encoding for the structural and enzymatic proteins required for viral replication and assembly of progeny virions (Figure 1.2). The Group specific antigen (Gag) is a 55 kDa polyprotein precursor that is composed of structural proteins required for virion assembly. The Gag polyprotein is cleaved by the viral protease into the mature proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (Freed 2001). The matrix protein is a 17 kDa myristoylated protein that, as the N-terminal end of the p55 Gag precursor, is responsible for localizing the Gag polyprotein to the plasma membrane for viral particle assembly (Bukrinskaya 2007). Mature matrix

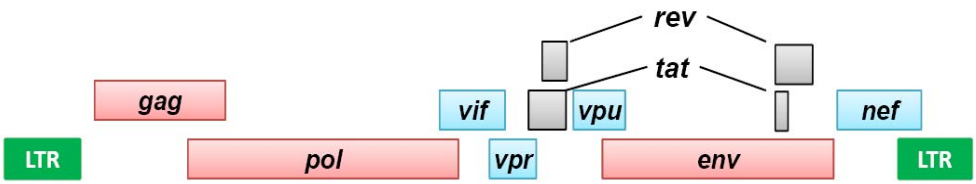

Figure 1.2 The genome of HIV-1.

Arrangement of the genes of HIV including the long terminal repeats (LTR) at both ends. The structural and enzymatic genes of HIV, *gag, pol*, and *env,* that are present in all retroviruses are shown in pink. The regulatory *tat* and *rev* genes are shown in grey and the four accessory genes *vif, vpr, vpu*, and *nef* in blue

protein is also part of the virions and it has been postulated to play an important role in transporting the viral reverse transcription complex to the nucleus of newly infected cells (Bukrinskaya, Brichacek et al. 1998). The capsid protein (p24) multimerizes to form the core of the virion, with approximately 2000 capsid molecules comprising the core of each viral particle (Frankel and Young 1998). The nucleocapsid protein is a 7 kDa protein required for packaging of the genomic viral RNA into nascent virions through an interaction with the packaging signal, the Ψ-site in the full length HIV RNA transcript (D'Souza and Summers 2005). The C terminal region of the Gag polyprotein is comprised of the p6 protein which has been shown to be involved in efficient viral particle release (Huang, Orenstein et al. 1995) as well in the incorporation of another viral protein, viral protein R (Vpr), into the viral particle (Checroune, Yao et al. 1995).

Polymerase (Pol) is also a polyprotein that is cleaved by autocatalysis into three mature proteins, Protease, Reverse Transcriptase, and Integrase that are the enzymatic components required for HIV replication. The viral protease cleaves the Gag polyprotein into its mature components, allowing the immature viral particles being produced to

become infectious as well as to maximize the efficiency of particle release (Kaplan, Manchester et al. 1994). Protease also cleaves Pol into its enzymatic components. Reverse Transcriptase (RT) is a heterodimer composed of a 51 kDa subunit and a 66 kDa subunit, both of which are derived from the Pol polyprotein (Frankel and Young 1998). The p66 subunit consists of the reverse transcriptase domain and an additional RNase H, at the C terminal end. The p51 subunit is derived from p66 with the 15 kDa RNase H domain cleaved by the viral protease (Freed 2001). Reverse Transcriptase is the polymerase that generates a cDNA copy of the viral genomic RNA. The viral RNase H degrades the RNA from the DNA-RNA hybrid, allowing RT to generate the complementary DNA to complete the double stranded DNA copy of the HIV genome. Importantly, Reverse Transcriptase does not possess a 3'-5' exonuclease activity for proofreading and has been reported to have error rates ranging from $2.16x10^{-5}$ to $3.4x10^{-5}$, contributing to the high genetic diversity of HIV (Mansky and Temin 1995; Keele, Giorgi et al. 2008). The viral Integrase is a 32 kDa protein that catalyzes the integration of the double-stranded viral DNA into the host cell genome following the completion of reverse transcription and localization of the viral DNA to the nucleus. Integrase cleaves nucleotides from the 3' end of each strand of the viral DNA and also makes a staggered cleavage of host cell genomic DNA. The viral

DNA is then joined to the ends of the cleaved cellular DNA and the integration completed by host cell DNA repair enzymes that fill in the gaps at the ligation sites (Freed 2001).

The envelope glycoproteins (Env) are also synthesized as a 160 kDa polyprotein which, unlike the Gag and Pol polyproteins, is cleaved and processed by cellular proteases (Moulard and Decroly 2000). The mature envelope glycoproteins are a 120 kDa surface protein glycoprotein 120 (gp120) that contains the determinants for interaction with cell surface receptor CD4 and co-receptor CCR5 or CXCR4 required for infection and a 41 kDa transmembrane protein glycoprotein 41 (gp41) that has domains that catalyze the fusion of viral and cellular lipid bilayers during virus entry (Freed 2001).

The regulatory proteins: Tat and Rev

In addition to the structural and enzymatic proteins present in all retroviruses HIV also encodes two regulatory proteins, trans-activator of transcription (Tat) and regulator of expression of viral proteins (Rev). These proteins are both expressed early in infection and have critical roles in viral transcription and replication. Although the HIV-1 promoter region

contains a number of sites for cellular transcription factors to help enhance the rate of transcription from the integrated provirus, the efficiency of elongation of these transcripts is rather low. Expression of the viral Tat protein increases the production of viral RNA by approximately 100 fold and has been shown to be required for viral replication (Dayton, Sodroski et al. 1986; Fisher, Feinberg et al. 1986; Frankel and Young 1998). Unlike the majority of cellular transcription factors that bind to the viral DNA, Tat binds to a stem-loop structure at the 5' end of the newly transcribed viral RNA known as the trans-activating response element (TAR). Tat then recruits Cyclin T1 and CDK9 which in turn phosphorylate RNA polymerase II increasing the efficiency of elongation of the nascent viral mRNA (Freed 2001).

Transcribed HIV RNA is primarily found in three different categories: 1) Unspliced RNA that serves as the mRNA for Gag and Pol as well as the genomic RNA that is packaged into virions; 2) singly spliced RNA that serves as the mRNA for Env and the accessory proteins Vif, Vpr, and Vpu; and 3) multiply spliced RNAs from which Tat, Rev and Nef are translated. Viral transcripts, like most cellular mRNAs, are spliced prior to being transported out of the nucleus, which allows expression of the viral regulatory proteins early in infection, preventing the efficient

export of the full length viral RNA for expression of Gag and Pol as well as for packaging. To export the unspliced viral RNA out of the nucleus, Rev is required. Multiple Rev molecules bind to a region in the env gene of the viral RNA that is only present in unspliced and singly spliced transcripts known as the Rev response element (RRE). Rev bound to the RRE then interacts with exportin-1 to form a nuclear export complex, to transport the unspliced and singly spliced viral transcripts to the cytoplasm (Pollard and Malim 1998).

The accessory proteins: Vif, Vpr, Vpu, and Nef

HIV-1 also encodes four accessory proteins which function to enhance viral replication and pathogenicity. The Viral infectivity factor (Vif) is a 23 kDa protein that is required for viral replication in vivo as well as in most cell lines. Cytosine deaminases APOBEC3G and APOBEC3F, both of which have been shown to restrict retroviral replication, are widely expressed in most cell types, including T cells (Harris and Liddament 2004). Vif allows HIV to evade host cell restriction factors APOBEC3G and 3F by targeting them for degradation through the proteosome pathway (Tian, Yu et al. 2006; Mehle, Wilson et al. 2007; He, Zhang et al. 2008). In the absence of Vif, APOBEC3G and 3F are encapsidated into

nascent virions and through their cytosine deaminase activity hypermutate the viral DNA during reverse transcription.

Viral protein R (Vpr) is a 96 amino acid protein that is incorporated into virions through an interaction with the viral p6 protein (Checroune, Yao et al. 1995). Vpr contains a nuclear localization signal (NLS) and following uncoating in a newly infected cell, Vpr is involved in transporting the pre-integration complex (PIC), which is the site of reverse transcription and contains the viral DNA/RNA as well as components of the capsid, to the nucleus of the cell. In addition to its nuclear localization function, Vpr also induces cell cycle arrest at the G2/M transition by binding to and inactivating CDC25C, a phosphatase, resulting in cyclin B1-p34Cdc2 remaining phosphorylated and in its inactive form (Romani and Engelbrecht 2009). The cell cycle arrest increases virus replication as transcription of the provirus is higher in G2. Vpr has also been associated with reducing the mutation rate of HIV (Mansky 1996). Vpr interacts with Uracil-DNA glycosylase 2 (UNG2) to incorporate it into nascent virions. UNG2 is a cellular DNA repair enzyme that removes uracil from DNA. Uracil can be introduced into DNA by either by misincorporation of dUTP or by deamination of cytosine. The recruitment of UNG2 into virions by Vpr has been associated with the reduction of mutations in the HIV genome (Chen, Wang et al. 2002).

Viral protein U (Vpu) is a 16 kDa membrane protein expressed late in infection. The most well characterized function of Vpu is the degradation of CD4 at the ER. Vpu binds to the cytoplasmic domain of CD4 in the ER and recruits an E3 ubitiquin ligase complex through an interaction with one of the components of the complex, βTrCP. The recruitment of the E3 complex results in the ubitiquination of CD4 which is then exported from the ER to a cytoplasmic proteosome for degradation (Margottin, Bour et al. 1998). In the absence of Vpu, the viral Env protein gp160 interacts with CD4 in the ER. This interaction with CD4 prevents the trafficking and maturation of gp160 (Nomaguchi, Fujita et al. 2008). Vpu is also important for the efficient release of viral particles, a function for which the mechanism has recently been discovered. CD317, a cell surface protein previously identified as Bone marrow stromal cell surface gene 2 (BST2), was found to tether nascent HIV viral particles to the cell surface resulting in the virions being endocytosed and not released. Vpu was found to co-localize with CD317, subsequently renamed Tetherin, and inhibit the retention of HIV particles at the cell surface (Neil, Zang et al. 2008).

Negative factor (Nef) is a 27-32 kDa myristoylated protein. A number of roles have been associated with Nef, with the four most well characterized functions being the downregulation of cell surface CD4, downregulation of major histocompatibility complex 1 (MHC-I), activation of p21-activated kinase 2 (PAK-2), and the enhancement of viral infectivity. The downregulation of cell surface CD4 by Nef has been shown be important for increasing the infectivity of nascent virions. CD4 on the cell surface interacts with HIV Env proteins at the plasma membrane, blocking efficient Env incorporation into viral particles while also causing some CD4 molecules to be incorporated into virions (Lama, Mangasarian et al. 1999). Downregulation of CD4 by Nef is proposed to be through a direct interaction and occurs independent of phosphorylation of serine residues on the cytoplasmic tail of CD4, unlike CD4 downregulation by phorbol esters which require serine phosphorylation (Garcia and Miller 1991). Nef expressing cells treated with lysosomal inhibitors resulted in CD4 accumulation in lysosomes, suggesting that downregulation of CD4 by Nef results in CD4 degradation in lysosomes (Luo, Anderson et al. 1996). The mechanism of Nef targeting CD4 for downregulation has been recently described. Nef, the cytoplasmic tail of CD4 and adapter protein-2 (AP-2) form a ternary complex that seems to target CD4 to the lysosome. This interaction involves a non-canonical

interaction between the Nef dileucine motif (EXXXLL) and AP-2. The weak interaction between the two leucines and AP-2 is not stabilized by the upstream glutamic acid, but by a downstream diacidic motif (Lindwasser, Smith et al. 2008). Downregulation of MHC-I by Nef is important for evading the host immune response by preventing the presentation of viral antigens to cytotoxic T cells. The mechanism of MHC-I downregulation by Nef also involves a non-canonical interaction with an adaptor protein which in this case is AP-1 (Noviello, Benichou et al. 2008; Wonderlich, Williams et al. 2008). Nef has also been shown to activate the cellular serine-threonine kinase PAK2 (Arora, Molina et al. 2000). While the role this activation plays in viral infection remains unclear, Nef from different HIV subtypes maintain this function using different interaction domains, suggesting that PAK2 activation is important for viral fitness (O'Neill, Kuo et al. 2006). Enhancement of viral infectivity by Nef in single round infectivity assays has also been shown. The exact mechanism by which Nef enhances virus infection remains poorly understood, but it has been shown to be independent of CD4 downregulation, as these *in vitro* assays were performed in cells lacking CD4 expression (Campbell, Nunez et al. 2004; Pizzato, Helander et al. 2007).

Reverse transcription of HIV genomic RNA

One of the defining hallmarks of HIV replication, as with all retroviruses, is the obligatory step of converting the single stranded viral RNA into a double-stranded DNA copy, referred to as reverse transcription, outlined in figure 1.3. The double stranded DNA form (provirus) consists of a minus strand and a plus strand that is the coding strand corresponding to the viral RNA sequence. Reverse transcription is dependent on the binding of a cellular $tRNA^{Lys3}$ to the primer binding site (PBS) at nucleotides 182-199 of the genomic viral RNA (Step 1). The viral enzyme Reverse Transcriptase (RT) initiates synthesis of the first strand of the viral DNA (minus strand) to the 5' end of the viral RNA (Step 2). As this DNA synthesis is progressing a component of the RT enzyme, RNase H, co-transcriptionally degrades the RNA portion of this DNA-RNA hybrid (Step 3). This segment of minus-strand DNA, known as the first strong stop DNA, then moves to the 3' end of the viral genome using the direct repeat (R) at the 3' end of the viral RNA (Step 4). This is referred to as the first strand transfer. The first strong stop DNA then serves to prime reverse transcription from the 3' end of the viral genomic RNA to completely transcribe the remaining viral DNA to the PBS (Step 5). As elongation of the minus-strand is occurring, RNase H co-transcriptionally

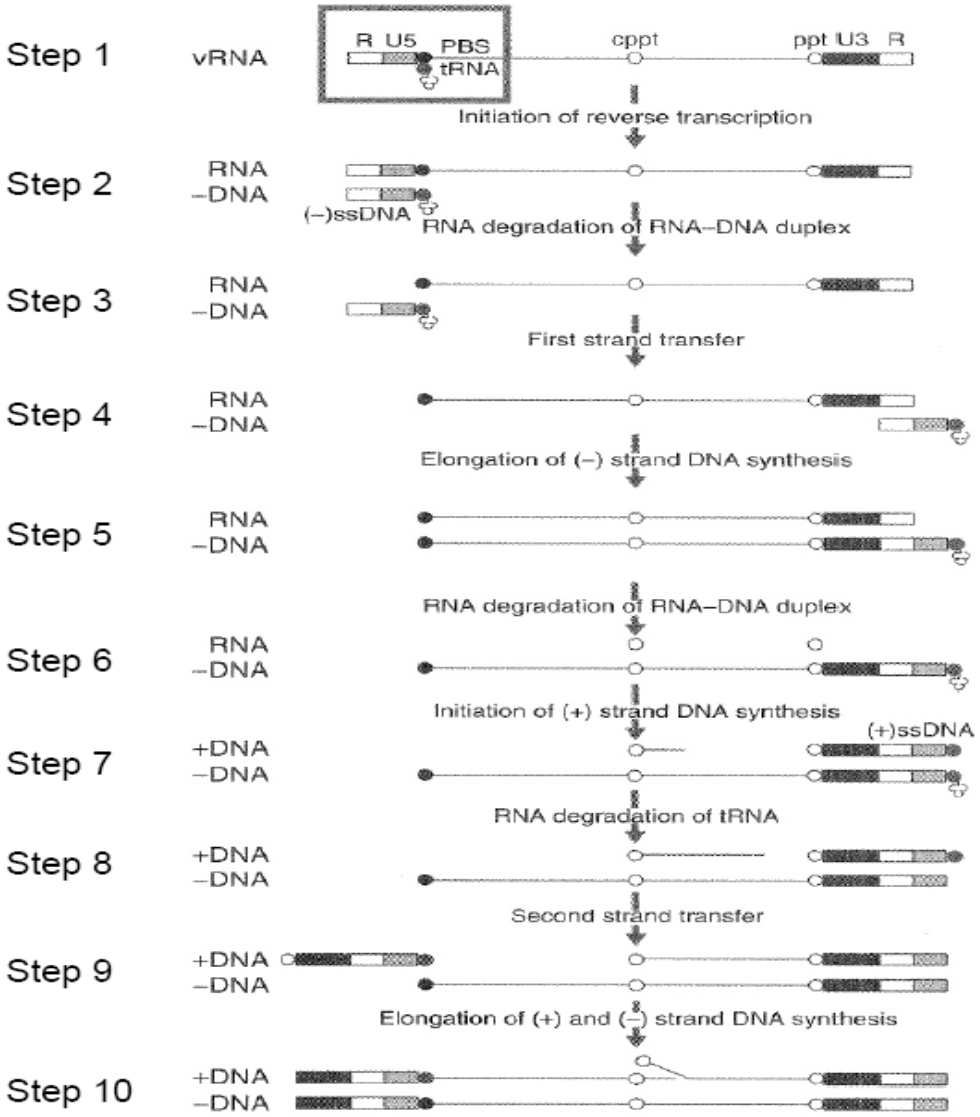

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Figure 1.3 Reverse transcription of HIV genomic RNA.

Diagrammatical representation of the steps involved in the process of converting the HIV genomic RNA into a double stranded provirus to be integrated into the host cell chromatin.

degrades the RNA in the DNA-RNA hybrid, which leaves temporarily single-stranded regions of the viral genome Step 6). Importantly, two short regions of the viral RNA remaining are uniquely resistant to degradation by RNase H. These two purine rich regions are known as the polypurine tracts (PPT). The first PPT is located in the pol region of the viral genome and is referred to as the central polypurine tract (cPPT) while the second is in the 3' region of the genome in the middle of the nef gene (3'PPT). The RNA from these two PPT's serves to prime synthesis for the plus DNA strand corresponding to the viral RNA sequence (Step 7). DNA synthesis from both PPTs completes the 3' half of the provirus, however when the cPPT primed DNA reaches the 3'PPT it displaces the previously replicated strand primed from the 3'PPT (Step 8). This is referred to as second strand transfer. This transferred strand consisting of U3, R, U5 and a DNA copy of PBS serves to prime plus strand synthesis of the 5' half of the provirus (Step 9). The final steps of reverse transcription include the generation of the 5' half of the plus strand and the 5'LTR of the minus strand (Step 10).

CHAPTER TWO: The APOBEC Family of Proteins

Identification of an antiviral factor antagonized by Vif

HIV is able to replicate in activated human peripheral blood mononuclear cells (PBMC) as well as in T cell lines. However, HIV lacking a functional *vif* gene is incapable of replicating in activated human PBMC. Surprisingly HIV *vif*⁻ was found to replicate in some T cell lines but not other. Specifically T cell lines, such as CEM and H9, do not support replication of Vif-deficient HIV and were subsequently termed non-permissive while other T cell lines, such as SupT1 and Jurkat, are permissive for HIV lacking Vif (Gabuzda, Lawrence et al. 1992). Furthermore, cell fusion experiments between permissive and non-permissive cell lines showed that the non-permissive phenotype is dominant in the heterokaryons, suggesting that non-permissive cells express an antiviral factor that is overcome by Vif (Madani and Kabat 1998). In 2002, a subtractive screen of RNA transcripts from non-permissive CEM cells that are not present in a permissive derivative cell line, CEM-SS, identified a potential candidate protein, termed CEM-15 (Sheehy, Gaddis et al. 2002). Importantly, when CEM-15 was expressed in permissive CEM-SS cells, it conferred a non-permissive phenotype,

blocking the replication of Vif-deficient HIV. Additionally, Sheehy et al noted the similarity between the amino acid sequence of CEM-15 and Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1).

The human APOBEC family of proteins

The APOBEC proteins are cytosine deaminases having a diverse range of functions. APOBEC1 is an RNA deaminase that is responsible for deaminating Apolipoprotein B (ApoB) mRNA at cytosine 6666, changing a glutamine to a stop codon (CAA to UAA), which results in a truncated form of the ApoB protein (Wedekind, Dance et al. 2003). A second member of the APOBEC family is the Activation induced deaminase (AID), a DNA deaminase expressed in germinal center B cells. AID is localized to the nucleus and deaminates cytosines on DNA of immunoglobulin genes during transcription resulting in increased antibody diversification (Goodman, Scharff et al. 2007). APOBEC2 is encoded on chromosome 6 and the protein is found to be expressed in cardiac and skeletal muscle cells (Liao, Hong et al. 1999). APOBEC2 has been shown to deaminate cytosines on both DNA and RNA *in vitro*, however, an *in vivo*

substrate and role for its expression have yet to be identified (Anant, Mukhopadhyay et al. 2001).

In 2002 a cluster of genes that shared homology to APOBEC1 was identified on chromosome 22 (Jarmuz, Chester et al. 2002). These genes were designated APOBEC3A-3G and have been hypothesized to have arisen by a series of gene duplication events, as primates have multiple APOBEC3 genes while other mammals such as mice have only one APOBEC3 (Wedekind, Dance et al. 2003; Harris and Liddament 2004). More recently an additional APOBEC3 was recognized, APOBEC3H (OhAinle, Kerns et al. 2006). The APOBEC3 proteins exclusively deaminate deoxycytosine on single stranded DNA. One of the APOBEC3 proteins, 3G was found to be identical to the HIV restriction factor CEM-15 (Jarmuz, Chester et al. 2002; Sheehy, Gaddis et al. 2002). Much research has been focused on the other APOBEC3 proteins to identify additional retroviral restriction factors. Shortly after the identification of APOBEC3G as an anti-HIV restriction factor, APOBEC3F was found to also have antiretroviral activity directed against HIV (Liddament, Brown et al. 2004; Wiegand, Doehle et al. 2004; Zheng, Irwin et al. 2004). Interestingly, all of the other APOBEC3 proteins have been reported to have various antiviral activities, however, the literature for APOBEC3A, 3B, 3C, 3DE, and 3H

have been conflicting as to their ability to restrict HIV (Yu, Chen et al. 2004; Doehle, Schafer et al. 2005; Dang, Wang et al. 2006; Bourara, Liegler et al. 2007; Goila-Gaur, Khan et al. 2007; Dang, Siew et al. 2008). In contrast, the antiretroviral activities of APOBEC3G and 3F are well documented and have been the focus of most of the anti-HIV APOBEC research in recent years.

Deaminase activity of APOBEC3G and APOBEC3F

The APOBEC3G and 3F deoxycytosine deaminase activity has been characterized in detail *in vitro,* specifically neither enzyme was found to act on single stranded RNA, double-stranded DNA, DNA-RNA hybrids. (Harris, Bishop et al. 2003; Zhang, Yang et al. 2003; Liddament, Brown et al. 2004). The target site specificity for cytosine deamination for each of the proteins does however differ, as APOBEC3F specifically targets cytosines at the nucleotide sequence 5'-HTC, where H is any nucleotide except guanine. This translates into a 5'-GAD to 5'-AAD mutation on the complementary strand. While APOBEC3G does also act on the 5'-HTC sequence, it preferentially targets the nucleotide sequence 5'-HCC, deaminating the 3' cytosine resulting in a 5'-GGD to 5'-AGD mutation in the complementary sequence (Harris, Bishop et al. 2003; Zhang, Yang et

al. 2003; Liddament, Brown et al. 2004). The specificity of APOBEC3G and 3F deamination sites generates signature mutations that allow identification of the enzyme responsible for the mutation.

APOBEC restriction of vif-deficient HIV

The best characterized antiviral mechanism of APOBEC3G, diagramed in figure 2.1, is dependent on its cytosine deaminase activity, although other mechanisms have been proposed. In a cell infected with HIV lacking a functional Vif, APOBEC3G and 3F become incorporated into the cores of nascent virions (Sheehy, Gaddis et al. 2002; Soros, Yonemoto et al. 2007). The amount APOBEC3G encapsidated is dependent on expression of the protein, but has been shown to be approximately 4-11 molecules of APOBEC3G per virion (Xu, Chertova et al. 2007). Upon entry into a new target cell, these APOBEC-containing virions uncoat and initiate reverse transcription, generating a complementary DNA strand (minus strand) to the genomic RNA. As the viral RNase H degrades the RNA from the DNA-RNA hybrid, regions of the minus strand of the viral genome are single-stranded making them substrates for exogenously packaged APOBEC3G (Harris, Bishop et al. 2003). Completion of the plus strand DNA synthesis renders the viral

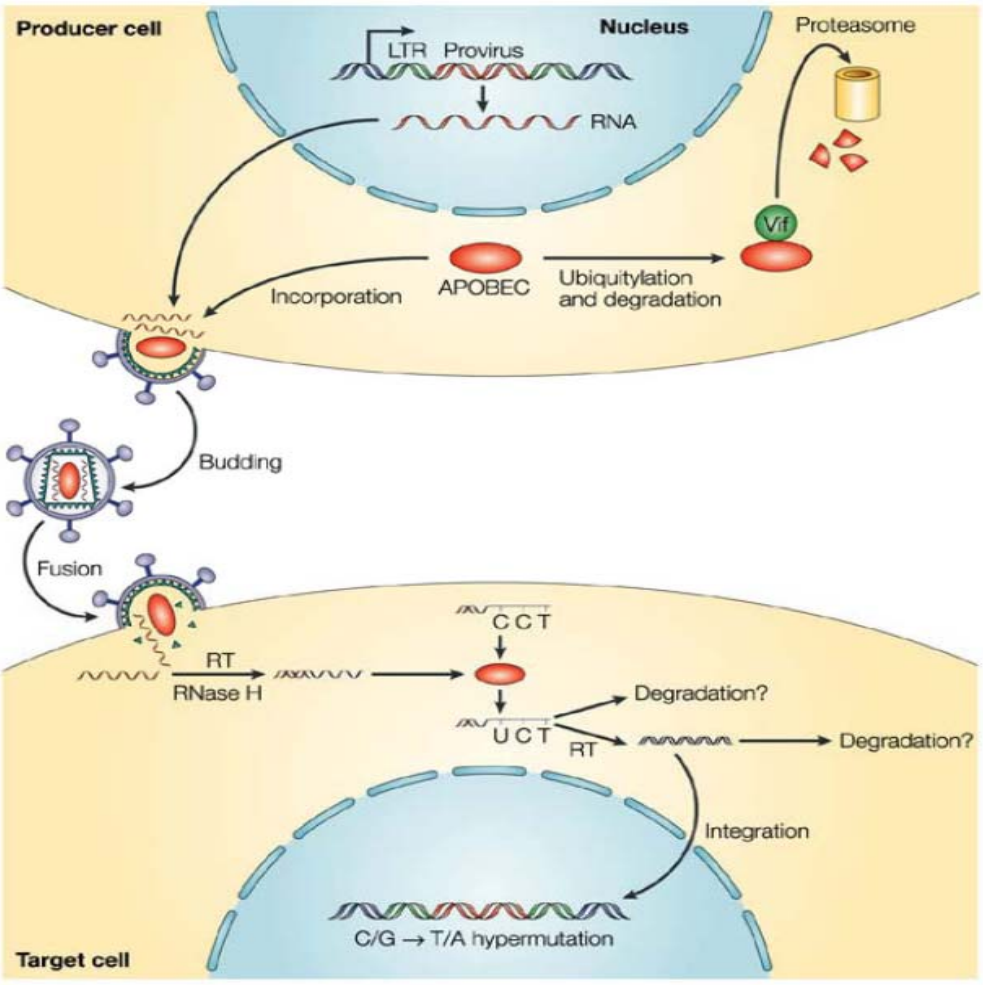


Figure 2.1 APOBEC restriction of HIV.

In an HIV infected cell in the absence of Vif, APOBEC becomes incorporated into the cores of nascent virions. Following infection of a new target cell, the HIV genomic RNA is reverse transcribed, leaving transiently single stranded DNA regions of the HIV genome which are the substrate for APOBEC deamination. Cytosines deaminated on the minus strand of the viral DNA are recognized as uracil, resulting in adenine being incorporated as the complementary base resulting in G to A mutations on the coding (plus) strand. Hypermutated HIV DNA is then either integrated into the host cell chromatin or degraded by host cell enzymes.

genome impervious to APOBEC3G mutation. Therefore, the deamination of cytosines on the minus strand causes the viral Reverse Transcriptase to recognize them as uracil and incorporate adenine as the complementary nucleotide during plus strand synthesis resulting in G to A mutations in the coding strand of the HIV provirus. The single-strand specificity of APOBEC3G and 3F correlates with an increased number of G to A mutations present in the genome of vif-defective HIV in the regions immediately 5' of the central polypurine tract (cPPT) and the 3' LTR polypurine tract (3'PPT), which remain single stranded the longest during reverse transcription (Yu, Konig et al. 2004; Suspene, Rusniok et al. 2006). The APOBEC induced deamination of the minus strand has been shown to severely affect virus viability. The deleterious mutational effect of APOBEC3G on the viral genome has been demonstrated *in vitro* (Mangeat, Turelli et al. 2003; Zhang, Yang et al. 2003).

An alternative outcome of the deamination of viral reverse transcripts other than the integration of lethally mutated provirus is the degradation of reverse transcripts by viral associated uracil DNA glycosylase-2 and apurinic/apyrimidinic endonuclease (UNG-2/APE1). UNG-2 excises uracils incorporated into DNA, leaving an abasic site which

is further processed by APE1 generating a 5'deoxyribose phosphate group to be repaired by DNA repair enzymes. The accumulation of uracil in HIV reverse transcripts processed by UNG-2 and cleaved by the endonuclease APE1 has been hypothesized to result in degradation of reverse transcripts (Harris, Bishop et al. 2003; Mangeat, Turelli et al. 2003). Importantly, this antiviral mechanism will not leave a mutational footprint that can be detected, as with integrated proviral DNA that has been lethally hypermutated.

Deamination of cytosines during reverse transcription of HIV can have extremely deleterious consequences for the virus. Mutations introduced into HIV in the context of the coding strand of the genome will occur at nucleotide sequences 5'-GGD and 5'-GAD where D is any base except C, resulting in the underlined G to be changed to A. The lethality of such mutations is highlighted in tryptophan (W) residues which have only one codon, TGG. Any G to A substitution will result in a termination codon (TAG, TGA or TAA). Only tryptophans followed by an amino acid whose codon begins with a C (leucine, proline, histidines, glutamine, or arginine) would not be targeted by APOBEC deamination. In addition to tryptophan codons, initiation codons are also potential substrates for APOBEC deamination. Any methionine followed by RDN, where R is

either G or A, D is any base except C, and N is any base, would be potentially subject to APOBEC deamination. A substitution of G to A in an initiation codon (ATG to ATA) results in a methionine to isoleucine substitution and loss of the start codon. Any initiation codon followed by isoleucine, asparagine, arginine, glycine, aspartic acid, glutamic acid, valine, or serine (AGT or AGC) is a putative APOBEC restriction site. In the HIV-1_{JR-CSF} genome, the initiation codons of 7 of the 9 genes are putative APOBEC restriction sites (*gag, pol, vif, vpr, tat, env and nef*). A loss of either Gag, Pol, or Env would be lethal for the virus, a feat that APOBEC proteins could accomplish with a single deamination event.

Antiviral activities of APOBEC3G and 3F that can be dissociated from their deaminase activity have also been reported (Newman, Holmes et al. 2005; Bishop, Holmes et al. 2006). Both deaminase active and deaminase defective APOBEC3G and APOBEC3F interfere with the initiation of reverse transcription as well as interacting with the viral protein Integrase to block proviral integration (Guo, Cen et al. 2006; Luo, Wang et al. 2007; Yang, Guo et al. 2007). The ability of deaminase defective APOBEC3G to restrict HIV is subject to debate, however, as increased amounts of the protein are required to achieve the antiviral effects. Two independent groups have demonstrated that when deaminase defective

APOBEC3G expression is titrated down to comparable amounts of the wild type protein, the antiviral effects of the deaminase defective APOBEC3G are negligible, while the wild type protein exhibits a marked restriction of HIV (Miyagi, Opi et al. 2007; Schumacher, Hache et al. 2008).

Vif recruits an E3 ubitiquin ligase complex

APOBEC restriction of HIV is blocked by the 192 amino acid viral protein Vif. Shortly following the identification of APOBEC3G as an antiviral factor that is countered by Vif, the mechanism of negation was found to be the proteosome pathway of degradation (Conticello, Harris et al. 2003; Marin, Rose et al. 2003; Sheehy, Gaddis et al. 2003; Yu, Yu et al. 2003). Subsequently it was determined that Vif also targets APOBEC3F for proteosomal degradation. Vif binds to APOBEC3G and 3F by overlapping but distinct domains in its N-terminal half (Tian, Yu et al. 2006; Mehle, Wilson et al. 2007; He, Zhang et al. 2008). Shown in figure 2.2, Vif also assembles a macromolecular E3 ubiquitination ligase complex consisting of elongin B, elongin C, Ring Box-2 (Rbx-2), Cullen 5 and an E2 (Stanley, Ehrlich et al. 2008). Vif binds Cullen 5 and elongin C at a 30 amino acid segment (144-173) with striking sequence similarity to

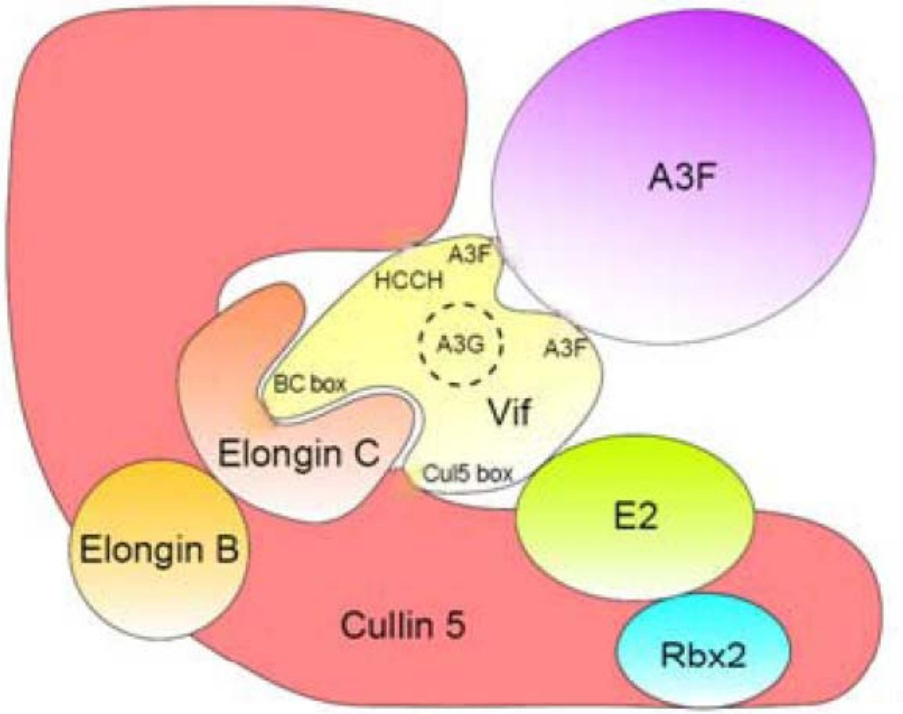


Figure 2.2 Vif targets APOBEC3G and 3F for degradation.

Vif (yellow) binds to APOBEC3F (purple) or APOBEC3G (not shown) through domains in its N-terminal half and recruits a ubitiquin ligase complex by binding of Cullen 5 (pink) and elongin C, targeting them for degradation through the proteosome pathway.

a suppressor of cytokine signaling (SOCS) binding domain. The SOCS domain contains a critical BC-box, SLQYLAAL (amino acids 144-153), that interacts with elongin C (Mehle, Goncalves et al. 2004). The C-terminal half of the SOCS binding domain has been shown to bind to Cullen 5 (Stanley, Ehrlich et al. 2008). Vif also interacts with Cullen 5 through a unique Zn binding region (amino acids H108, C114, C133, H139 (Mehle, Thomas et al. 2006; Xiao, Xiong et al. 2007). Rbx-2 binds directly to Cullen 5 and this complex recruits E2. Whether Vif binds to APOBEC3G and 3F prior to recruiting the ubitiquin ligase complex is not known but these proteins have been shown to be ubiquitinated. Additionally, Vif is polyubiquitinated in the presence of APOBEC3G and is also degraded by the proteosome pathway (Mehle, Strack et al. 2004).

Vif binding to APOBEC3G and APOBEC3F is functionally separable

Vif binds to APOBEC3G and 3F by overlapping but distinct domains in its N-terminal half (Tian, Yu et al. 2006; Mehle, Wilson et al. 2007; He, Zhang et al. 2008). The interaction between Vif and

APOBEC3G has been localized to amino acids 40-71, with the mutation of histidines 42 and 43 to asparagines disrupting binding to APOBEC3G but not affecting Vif binding APOBEC3F (Mehle, Wilson et al. 2007). This Vif-APOBEC3G interaction site has been further divided into amino acids 40-44 being specific for APOBEC3G, amino acids 52-72 involved in binding both APOBEC3G and APOBEC3F, and amino acids 74-79 specific for APOBEC3F (He, Zhang et al. 2008). In addition to amino acids 74-79 of Vif being required for APOBEC3F interaction, point mutations in amino acids 11-17 have been shown to disrupt APOBEC3F binding while allowing Vif to remain effective against APOBEC3G (Yamashita, Kamada et al. 2008).

Species specificity of the Vif-APOBEC interaction

Restriction of retroviruses by APOBEC3G and 3F is conserved in multiple primate species; however the ability of Vif to interact with APOBEC is species specific. While HIV-1 Vif is capable of targeting both human APOBEC3G and 3F for degradation, APOBEC3G from African green monkey (AGM) is resistant to HIV Vif and thus blocks infection of AGM cells with HIV (Mariani, Chen et al. 2003). Conversely, Vif from AGM Simian immunodeficiency virus (AGM SIV) can inhibit AGM

APOBEC3G yet cannot neutralize human APOBEC3G (Bogerd, Doehle et al. 2004). This species specificity has been mapped to a single amino acid in APOBEC3G at residue 128. In AGM APOBEC3G, amino acid 128 is a lysine, while the same residue in human APOBEC3G is an aspartic acid. When the human APOBEC3G is changed to lysine at position 128, it becomes resistant to HIV Vif, but susceptible to Vif from AGM SIV. Likewise, substitution of the positively charged lysine at residue 128 in AGM APOBEC3G to a negatively charged aspartic acid makes it susceptible to HIV Vif, while conferring a resistance to AGM Vif (Bogerd, Doehle et al. 2004; Schrofelbauer, Chen et al. 2004).

In vitro escape of APOBEC3G by Vif-defective HIV

A recent *in vitro* study attempted to identify second site mutations that would allow HIV defective in Vif to replicate in APOBEC3G expressing cells. In this study, HIV with two stop codons at amino acids 26 and 27 to disrupt *vif* was propagated in a permissive cell line transduced to express APOBEC3G but not APOBEC3F. In 3 of 48 experiments, HIV lacking Vif was found to be able to replicate in the presence of APOBEC3G, despite a low level of G to A mutations occurring (Hache, Shindo et al. 2008). In all three escape mutants, the vif open reading frame remained disrupted,

however two specific mutations were attributed to the virus being able to replicate in the presence of APOBEC3G. An A to C/T mutation at nucleotide 200 in the 5' LTR was identified in all three escape mutants that was subsequently reported to eliminate an initiation codon upstream of the *gag* coding sequence thereby increasing translation efficiency of the viral mRNA (Hache, Abbink et al. 2009). It should be noted that A200 is not present in other strains of HIV, meaning A200 represents a partially replication defective mutation. The second mutation permitting replication in the presence of APOBEC3G was a nonsense mutation inactivating another accessory gene, *vpr*. The authors hypothesized that Vpr may play a role in facilitating APOBEC3G restriction, possibly by incorporating a cellular factor into HIV, thus the loss of Vpr permitted the virus to escape APOBEC3G restriction. The identity of the host cell factor remains unknown, nor is it clear why Vpr would incorporate such a factor into the virion. Importantly, these three escape viruses remained susceptible to restriction by other APOBEC proteins as they were not able to replicate in PBMC or in cells expressing APOBEC3F (Hache, Shindo et al. 2008).

In vivo analysis of SIV Vif function

HIV only infects humans, presenting a major obstacle for *in vivo* studies. As a result, model systems using related simian viruses are utilized to gain insight into *in vivo* activities of HIV. In one study, SIVmac239 was used to infect rhesus macaques. Prior to the identification of APOBEC3G as a retroviral restriction factor that is negated by Vif, a group attempting to identify attenuated mutants of SIV for potential vaccine candidates exposed rhesus monkeys to SIV with different accessory genes disrupted. One such mutant was defective in Vif, with only the first 28 amino acids present. In all three rhesus monkeys exposed intravenously to Vif-deficient SIV, no viral DNA was found in PBMC collected at 2 and 16 weeks post exposure and plasma from the monkeys showed little to no antibody against SIV antigens (Desrosiers, Lifson et al. 1998). The authors concluded the loss of Vif is extremely deleterious to SIV, but that low levels of viral replication occurred since weak antibody responses had been detected.

In a more recent *in vivo* study, a chimera between SIVmac239/HIV (SHIV) with a two amino acid change in the conserved SLQYLA motif of Vif was used to infect pigtail macaques. This SHIV was previously shown to be highly pathogenic and produce an AIDS-like disease. This two amino acid change (SLQYLA to AAQYLA) disrupts the interaction between Vif and elongin C to prevent recruitment of the ubitiquin ligase complex, but does not interfere with Vif binding to either APOBEC3G or 3F. In all three macaques exposed, attenuated viral replication occurred as viral RNA present in the plasma was at a 100 fold lower level compared to macaques infected with the parental virus (Schmitt, Hill et al. 2008). Additionally, $CD4^+$ T cell levels in the monkeys inoculated with the Vif mutant SHIV were not significantly reduced and no evidence of disease progression was observed. Analysis of the tissues of the Vif-mutant SHIV infected macaques revealed a reduced distribution of viral DNA in tissues compared to the parental SHIV infected macaques. Interestingly, G to A mutations characteristic of APOBEC proteins were present in the viral DNA, with the majority of the mutations in the context of 5'-TC on the minus strand, suggesting APOBEC3F was responsible for the majority of the mutations (Schmitt, Hill et al. 2008). This study raises a number of interesting questions regarding the effectiveness of APOBEC3F restriction and mechanisms of APOBEC3G inhibition by Vif.

The Vif/APOBEC axis as a potential therapeutic target

A significant interest in the Vif-APOBEC axis as a potential therapeutic target for HIV infection has developed in recent years. Our new understanding of the antiviral activity of APOBEC3G and 3F make the concept of therapeutically targeting Vif an appealing option. This approach will suppress viral replication by a different mechanism than current antiretroviral therapies. Inactivation of Vif would allow a potent arm of the innate immune system to attack the replicating virus, ultimately resulting in populations of hypermutated viral DNA that is no longer replication competent. Conversely, the error prone nature of HIV-1 Reverse Transcriptase results in high genetic diversity that could lead to the development of escape mutations allowing the virus to evade APOBEC restriction. Previous work in cell culture based systems with ectopically expressed APOBEC3G has demonstrated Vif-deficient HIV-1 can develop second site mutations to avoid restriction by a single APOBEC protein (Hache, Shindo et al. 2008), however, the ability of HIV to escape restriction by all APOBEC proteins *in vivo* remains to be addressed.

CHAPTER THREE: Methodology

Cell Culture

TZM Hela cells obtained from the NIH AIDS Research and Reference Reagent Program (Platt, Wehrly et al. 1998) and human embryonic kidney (HEK) 293T cells were cultured at 37^{o}C, 10% CO_2 in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% fetal bovine serum (SAFC Biosciences), 50 IU penicillin, 50 µg/ml streptomycin and 2mM L-glutamine (Cellgro). CEM-SS cells, a human T cell line, was cultured at 37^{o}C, 5%CO_2 in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (SAFC Biosciences), 50 IU penicillin, 50 µg/ml streptomycin, 2mM L-glutamine (Cellgro), and 1mM sodium pyruvate (Cellgro).

Transfections

HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. Cells, 7x10^{5} per well in a 6 well plate or 5x10^{6} cells in a 10cm dish, were cultured in DMEM in the absence of antibiotics 24 hours prior to transfection. Plasmid DNA was resuspended in Opti-MEM (Gibco) at a ratio of 1µg of DNA to 2.5µl of

Lipofectamine 2000. Following a 20 minute incubation at room temperature, the DNA-Lipofectamine 2000 mixture was added drop wise to the 293T cells.

Preparation of whole cell lysates and western blotting

Transfected 293T cells were washed with cold PBS while attached to the culture dish and then lysed in 800µl of cold lysis buffer [50mM Tris pH 8.0, 10% glycerol (Fisher), 100mM NaCl (Fisher), 25mM NaF (Sigma), 2mM Na_3VO_2 (Sigma), 20mM β-glycerophosphate (Sigma), 25mM benzamidine (Sigma), 2mM EDTA pH 8.0, and 0.5% IGEPAL CA630 (Sigma)] supplemented with 1mM phenylmethylsulphonyl fluoride (PMSF) (Sigma) and 1 mini protease inhibitor tablet (Roche) per 10ml of lysis buffer. Cell lysates were cleared by centrifugation at 15,000xg for 10 minutes at 4^{o}C in an eppendorf 5415R centrifuge and protein concentrations of the post-nuclear supernatants determined by spectrophotometry using IgG protein standards in Bio-Rad Protein Assay reagent (BioRad). Total protein, 200µg, from whole cell lysates in 1x sodium dodecyl sulfate (SDS) loading buffer were denatured for 5 minutes and resolved on a 12% polyacrylamide gel for 900 volt-hours. Proteins were then transferred to a Hybond-C Extra nitrocellulose membrane

(Amersham Biosciences) using a TransBlot semi-dry Transfer Cell (BioRad) at 20 volts for 42 minutes. The membrane was then blocked for 1 hour in 10% milk in Tris-Tween-Buffered Saline (TTBS) (10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween 20 (Fisher Scientific), incubated with primary antibody in 10% milk in TTBS for 12-16 hours at 4^{o}C, washed three times in TTBS, incubated with HRP-conjugated secondary antibody in 10% milk in TTBS for 2 hours, and finally washed three times in TTBS. To detect the HRP-conjugated antibodies, chemi-luminescence was used. The membrane was incubated in the luminol substrate [100 mM Tris pH8.8, 2.5 mM luminol (Fluka), 400 µM p-coumaric acid (Sigma), and 5.4 mM H_2O_2(Sigma)] for 5 minutes and then exposed to film (Fujifilm) for an appropriate amount of time to visualize the protein of interest. Films were developed using an automated developer system (AFP Imaging).

Antibodies

Rabbit anti Vif (#2221) obtained from the NIH AIDS Research and Reference Reagent Program (Goncalves, Jallepalli et al. 1994) was used at 1:1000. Mouse anti-HA.11 (clone 16B12 Covance MMS-101P) was used at 1:1000. Sheep anti-Nef was used at 1:1000 (Arora, Molina et al. 2000; Foster, Molina et al. 2001). Goat anti-rabbit horseradish-

peroxidase (HRP) (Chemicon AP307P) was used at 1:10,000. Rabbit anti-sheep (Upstate 12-342) was used at 1:10,000. Goat anti-mouse HRP (Zymed 81-6520) was used at 1:10,000.

Generation of vif-deficient HIV-1

All experiments were performed using HIV-1_{JR-CSF} obtained from the NIH AIDS Research and Reference Reagent Program (Koyanagi, Miles et al. 1987). To generate HIV defective in Vif, a single nucleotide insertion was introduced into the *vif* gene in the proviral DNA, HIV-1_{JR-CSF}vif(FS), by site directed mutagenesis. The location of the insertion was 86 nucleotides into the *vif* gene and did not alter the *pol* or *vpr* genes, both of which overlap *vif*. A second Vif deficient HIV-1 consisting of a 172 base deletion in the *vif* gene from nucleotides 5135 to 5307, HIV-1_{JR-CSF}vif(Del), was constructed by digesting HIV-1_{JR-CSF} proviral DNA with NdeI and PflmI (New England Biolabs), filling in the overhanging ends with DNA Polymerase I (Klenow) (New England Biolabs) and ligating the blunt ends with T4 DNA Ligase (New England Biolabs). As with the HIV-1_{JR-CSF}vif(FS), the location of the deletion does not affect the overlapping *pol* or *vpr* genes.

Production of HIV-1 stocks and Titration

HIV-1 stocks were generated by transfecting proviral DNA into 293T cells using Lipofectamine 2000 (Invitrogen) as indicated above. The virus containing culture supernatant was collected and cleared of cellular debris first by centrifugation at 3000 RPM for 20 minutes and then by filtering through a 0.45μm filter (Millipore). Infectious titers were determined by using TZM cells, a reporter cell line that expresses β-galactosidase under the control of the HIV-1 LTR. The viral Tat protein expressed in HIV infected cells induces β-galactosidase production resulting in infected cells turning blue upon staining with X-gal (5-bromo-3-indoyl-β-D-galactopyranoside) (Sigma). Blue cells were counted by microscopy and the counts were used to calculate the titer which was defined as tissue culture infectious units (TCIU).

Generation of CCR5 expressing T cell line

CEM-SS cells were transduced to express CCR5 with a retroviral vector. The vector was generated by co-transfecting 293T cells with pBabe-CCR5 obtained from the NIH AIDS Research and Reference Reagent Program (Morgenstern and Land 1990; Deng, Liu et al. 1996) and the packaging vector pEQPAM using Lipofectamine 2000 (Invitrogen).

The culture supernatants were collected after 48 hours and filtered through a 0.45μm filter. Twenty-four well plates were coated with 40 μg of Retronectin (Takara) and then washed with PBS+2% BSA and incubated twice with 0.5mL of the vector supernatants for one hour each. CEM-SS cells ($3x10^5$) were then incubated in the vector coated wells overnight at 37°C, 5%CO_2. The following day, the vector supernatant (0.5mL) was added to the cells overnight. Transduced cells were selected in complete RPMI containing 0.5 μg/ml puromycin. Fluorescence activated cell sorting was used to obtain a $CD4^{Hi}CCR5^{Hi}$ population. Briefly, antibodies to CD4 and CCR5 with fluorescent conjugates PE and APC respectively (Becton-Dickinson) were used to stain $1.5x10^7$ cells. The stained cells were then sorted on a BD FACSAria (Becton-Dickinson), collecting the top 25% of the $CD4^+CCR5^+$ population.

Viral Cultures

CCR5 expressing CEM-SS cells were used to propagate both wild type and vif-deficient HIV-1_{JR-CSF}. Cells ($1x10^6$) were infected in a 24 well plate with $5x10^4$ TCIU of virus in complete RPMI containing 4 μg/ml polybrene at 37° C, 5% CO_2 for 4 hours. The cells were then washed extensively with PBS and cultured at 37° C, 5% CO_2 in complete RPMI

containing 0.5 µg/ml puromycin to maintain selection of CCR5 expressing cells. Cell cultures were passaged every three days and a sample of the culture supernatant was collected for quantitation of the amount of viral capsid protein by p24 ELISA.

Generation of humanized NOD/SCID IL2γ$^{-/-}$ mice

Humanized NOD/SCID IL2γ-/- (NSG; NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ; The Jackson Laboratory Stock# 005557) mice were generated essentially as described by Traggiai et al (Traggiai, Chicha et al. 2004). Briefly, newborn pups were preconditioned by gamma irradiation with 100 rads 3 to 12 hours prior to intrahepatic transplant of $3x10^5$ $CD34^+$ human hematopoietic stem cells (HSC) isolated from human fetal liver as previously described (Melkus, Estes et al. 2006). $CD34^+$ cells were isolated using immunomagnetic beads (Miltenyi Biotech). Reconstitution of a human immune system in the mice was routinely monitored by flow cytometric analysis of peripheral blood of the transplanted mice.

Flow cytometry

Peripheral blood obtained from the humanized mice (50 µl) was first blocked with murine IgG (Sigma), and then stained with mouse anti-human CD45 (clone 2D1, Becton-Dickinson 557873) to exclude murine cells from the analysis. Human lymphocytes were identified by their human $CD45^+$ expression and further characterized by staining with mouse anti-human CD3 (clone HIT3a, Becton-Dickinson 555573). Human $CD3^+$ cells were further analyzed for $CD4^+$ and $CD8^+$ expression with mouse anti human CD4 (clone RPA-T4, Becton-Dickinson 555749) or CD8 antibodies (clone SK1, Becton-Dickinson 347314). Data was collected using a FACSCanto instrument and analyzed using FACS Diva software (Becton-Dickinson).

Quantitative RT-PCR of human APOBEC3G from humanized NOD/SCID IL2γ$^{-/-}$ mice

Copies of human APOBEC3G and APOBEC3F RNA in the different tissues were assessed in the humanized NOD/SCID IL2γ$^{-/-}$ mice by isolation of mononuclear cells from the tissues and purification of the cellular RNA using the RNeasy kit (Qiagen) according to the manufacturer's recommended protocol. All RNA samples were treated

with DNase (Qiagen) to eliminate any contaminating genomic DNA not removed by the RNeasy columns. Total RNA was then used as the template in a one step RT-PCR reaction with the TaqMan RNA-to-Ct 1 step kit (Applied Biosystems). Primers for the APOBEC3G and APOBEC3F RT-PCR were previously published (Vetter, Johnson et al. 2009) and are listed in table 3.1. APOBEC3G and APOBEC3F RNA for a standard curve for absolute quantitation was generated by *in vitro* transcription using a commercially available kit (Promega) from the T7 promoter of an APOBEC3G expression vector obtained from the NIH AIDS Research and Reference Reagent Program (Sheehy, Gaddis et al. 2002; Stopak, de Noronha et al. 2003). Control RNA concentrations were determined by spectrophotometry and used to calculate absolute copy number based on the length of the transcript and ten-fold serial dilutions used to generate the standard curve for quantitation.

Infection of humanized NOD/SCID IL2γ$^{-/-}$ mice with HIV-1

Humanized NOD/SCID IL2γ$^{-/-}$ mice were inoculated with either $9x10^4$ or $3.6x10^5$ tissue culture infectious units of wild type HIV-1_{JR-CSF} or vif-deficient HIV-1_{JR-CSF} intravenously by tail vein injection.

Peripheral blood analysis

Peripheral blood was collected from humanized mice from the retro-orbital vein using EDTA containing capillary tubes (Drummond Scientific). Whole blood was centrifuged and the plasma removed for analysis of viral antigenemia and viral RNA. The volume of plasma removed was replaced with solution B [1 liter PBS (Sigma) , 5g BSA (Sigma), 50U/ml penicillin (Sigma), 50mg/ml streptomycin (Sigma), 1% citrate phosphate dextrose (Sigma)]. The cellular portion of the blood was used for flow cytometric analysis as previously described or for genomic DNA isolation. Peripheral blood to be used for DNA isolation was resuspended in 1ml of a hypotonic red blood cell lysis buffer [500ml dH_2O, 4.15g NH_4Cl (Sigma), 0.5g $KHCO_3$ (Sigma), and 0.019g EDTA (Sigma)]. Following a 10 minute incubation at room temperature, the lysed blood was centrifuged at 10,000 RPM for 1 minute and the lysis buffer removed by aspiration. The resulting cell pellet was stored at -80^{o} C until DNA extraction.

Viral antigenemia analysis

The capsid protein of HIV-1, Gag p24, was used as the marker for viral antigenemia in the plasma of the humanized mice and as the indicator for viral replication in culture supernatants. HIV-1 p24 was detected and quantitated from the plasma of the humanized mice by ELISA using a commercially available kit (Beckman-Coulter) and from the viral culture supernatants by ELISA using a commercially available kit (Advanced Biosciences) both according to the manufacturer's protocol.

Plasma viral load analysis

Plasma viremia was determined by quantitating the copy number of cell free viral RNA. Plasma from the humanized mice, 20 µl was used to isolate viral RNA using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's protocol. Viral RNA was then quantified using a one step RT-PCR protocol. Isolated RNA from the plasma, 5 µl, was used as the template with the TaqMan RNA-to-Ct 1 step kit (Applied Biosystems). Primers for the RT-PCR (table 3.1) were previously published (Palmer, Wiegand et al. 2003) were directed against a conserved region of the *gag* gene.

Tissue harvest

The spleen, lymph nodes, and thymus were harvested and disrupted by grinding the tissue through a 70μm cell strainer (Falcon) with the plunger of a 3cc syringe and cells washed through the strainer with cold solution B. Red blood cells from the spleen were lysed with hypotonic lysis buffer and cells from all tissues pelleted by centrifugation at 1500 RPM for 5 minutes at 4^{o}C.

Mononuclear cells were isolated from the bone marrow by crushing the long bones from the hind legs using a mortar and pestle, collecting the cells by washing with cold solution B, and filtering the cells through a 70μm cell strainer. Red blood cells were lysed with hypotonic lysis buffer and cells pelleted by centrifugation at 1500 RPM for 5 minutes at 4^{o}C.

Mononuclear cells were isolated from the liver by grinding the tissue through a 70μm cell strainer and pelleting the cells by centrifugation at 1500 RPM for 5 minutes at 4^{o}C. The resulting cell pellet was then resuspended in 40% percoll and under layered with 70% percoll and centrifuged at 2400 RPM for 20 minutes at room temperature. Mononuclear cells were collected from the interface and washed with cold solution B.

Cells were isolated from the lungs by first mincing the tissue with scissors and digesting the tissue in 2.5 ml RPMI containing 6 mg

collagenase D (Roche) and 50 µg DNase I (Roche) for 30 minutes at 37^{o}C. The digested tissue was then ground through a 70µm cell strainer with a 3cc syringe plunger, washed though with cold solution B, and centrifuged at 1500 RPM for 5 minutes at 4^{o}C. As with the liver, mononuclear cells were isolated by resuspending the cell pellet in 40% percoll, under layering with 70% percoll, and centrifugation at 2400 RPM for 20 minutes at room temperature and the cells collected from the interface and washed with cold solution B.

Live cells from all tissues were counted by trypan blue exclusion and pelleted for genomic DNA isolation by centrifugation at 10,000 RPM for 1 minute or pelleted by centrifugation at 1500 RPM for 5 minutes and resuspended in freezing media [90% Heat-inactivated FBS, 10% dimethyl sulfoxide (DMSO) (Fisher)] and frozen live in a cryo-freezing container at -80^{o}C and then stored in liquid nitrogen.

Genomic DNA isolation and nested PCR of viral DNA

Genomic DNA was isolated from frozen peripheral blood cell pellets by resuspending the pellet in 0.01M Tris ph7.4 containing recombinant PCR grade proteinase K (Roche) and incubating at 56^{o}C for 1 hour. The proteinase K is then heat inactivated at 95^{o}C for 20 minutes and the

mixture frozen at -20^{o}C. After thawing, the samples are centrifuged for 1 minute at 14,000 RPM and the DNA containing supernatant transferred to a new unautoclaved eppendorf tube. Genomic DNA from mononuclear cells, 5x10^{5}-5x10^{6}, from animal tissues was prepared using QIAamp DNA blood mini columns (Qiagen) according to the manufacture's protocol.

Viral DNA was detected by nested PCR of a 741 nucleotide region of the 3' viral genome including the *nef* gene (HIV-1$_{JR-CSF}$ nucleotides 8721-9462). All PCR was performed using the Expand High Fidelity PCR System (Roche). The primers for the outer and inner reaction are listed in table 3.2. The reaction conditions were: a one-time denaturation at 95^{o}C for five minutes followed by 30 cycles of 95^{o}C for 1 minute, 55^{o}C for 1 minute, 72^{o}C for 1 minute. Amplification concluded with an elongation step at 72^{o}C for 7 minutes. A 1.4kB region from nucleotide 4947 through 6372 of the HIV-1$_{JR-CSF}$ genome that includes the *vif* gene was also amplified with the primers listed in table 3.2. The reaction conditions were: a one-time denaturation at 95^{o}C for five minutes followed by 30 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72oC for 1.5 minutes. Amplification concluded with an elongation step at 72^{o}C for 7 minutes. A 1.2 kb region of the viral genome from nucleotides 2562-3831 including the reverse transcriptase region of the *pol* gene was also performed. The primers for the outer and inner reaction are listed in table

3.2. The reaction conditions were: a one-time denaturation at 95^{o}C for five minutes followed by 30 cycles of 95^{o}C for 1 minute, 55^{o}C for 1 minute, 68^{o}C for 1 minute. Amplification concluded with an elongation step at 72^{o}C for 7 minutes.

Amplification of cell-free viral RNA

Viral RNA was isolated from the plasma of HIV-1_{JR-CSF} exposed humanized mice using a QIAamp viral mini kit according to the manufacturer's recommended protocol (Qiagen #52904). Purified viral RNA was then reverse transcribed into a cDNA with the reverse Nef outer primer (Table 3.2) using SuperScript III Reverse Transcriptase (Invitrogen #18080-044) according to the manufacturer's recommended protocol. The viral RNA was then digested using RNase (18021-014) and the cDNA used as a template in nested PCR reactions as described above.

DNA sequencing and analysis

Amplified viral DNA was sequenced using the inner nested PCR primers as sequencing primers. All DNA sequencing was done at the McDermott Center for Human Growth and Development at the University of Texas- Southwestern Medical Center. All chromatographs of the

amplified DNA sequences were visually analyzed and the nucleotide sequences aligned against the corresponding proviral DNA sequence used to generate the viruses using the Highlighter program which can identify G to A mutations indicative of APOBEC mutagenesis. Highlighter is available online through the Los Alamos National Laboratory at http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html. Highlighter output uses a graphical representation of DNA sequences for comparison (Figure 3.1). To assess the effects of G to A transition mutations on the viral protein sequence, Amplified DNA sequences were translated using the online ExPASy translation tool at http://au.expasy.org/tools/dna.html.

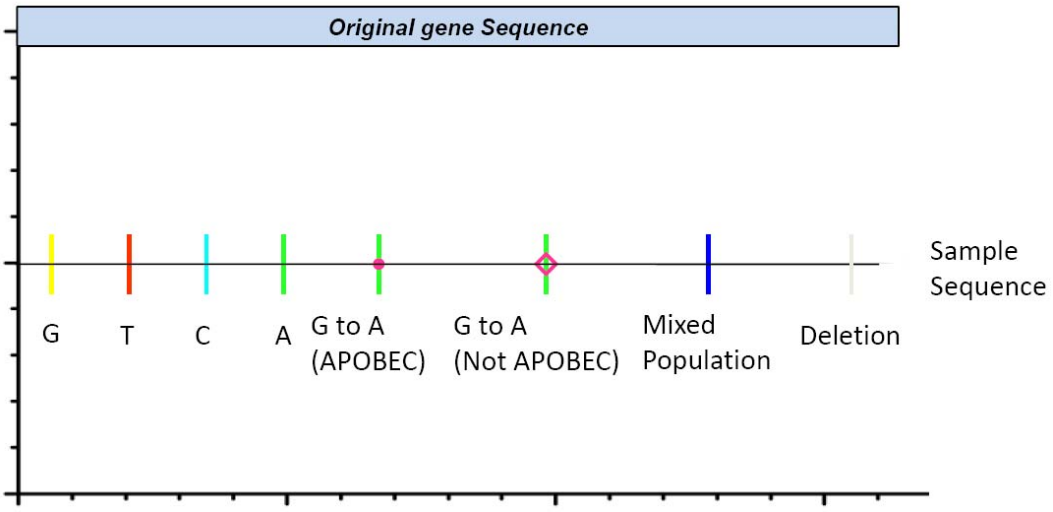


Figure 3.1 NIH highlighter sequence alignment program graphical output.

The top line in each graph represents the master sequence to which all other sequences are compared. Sample DNA sequences are represented by lines below the master sequence. Any change from the master sequence in DNA samples is shown as a perpendicular color coded line through the sample sequence. A nucleotide changed to a guanine is represented by a yellow line, a change to a thymine is a red line, a change to a cytosine is a light blue line, and a change to an adenine is a green line. Any G to A change at a putative APOBEC3G or 3F site (5'-GRD) is depicted by a green line with a pink circle while a G to A change not at an APOBEC site is shown as a green line with an open pink diamond. Any nucleotide change using an IUPAC code, such as R or N, is depicted by a dark blue line. Deletions are shown as a grey line.

Virus rescue assay

Cells from infected tissues were co-cultured with activated human peripheral blood mononuclear cells (PBMC) to detect the presence of replication competent virus. PBMC were obtained from HIV seronegative human donors and were activated for 72 hours with phytohemagglutin (PHA) (1µg/ml) in RPMI supplemented with 20% heat-inactivated FBS, 50 IU penicillin, 50 µg/ml streptomycin, 2mM L-glutamine, and 1mM sodium pyruvate. Following activation, activated PBMC, $1x10^6$, were cultured with mononuclear cells, $1x10^6$, isolated from HIV infected tissues of the humanized mice that were found to be positive for viral DNA by nested PCR analysis. These co-cultured cells were maintained in RPMI supplemented with 20% heat-inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2.5 µg/ml Fungizone, and 20U/ml interleukin-2 (Sigma) and virus replication was monitored by p24 ELISA analysis of the culture supernatant. Media from the culture was removed at multiple time points up to 21 days after culture initiation with an equal volume of fresh media replaced.

Table 3.1 Primers sequences for APOBEC qRT-PCR analysis

Region Amplified	Primer	Primer Sequence
Human APOBEC3G 1042-1110	5' A3G	5′-CTGCTGAACCAGCGCAGG-3′
	3' A3G	5′- GCGGCCTTCAAGGAAACC-3′
	Probe	5′-CTTTCTATGCAACCAGGCTCCACATAAAC-3′
Human APOBEC3F 833-909	5' A3F	5′-GCACCGCACGCTAAAGGA-3’
	3' A3F	5′-TTTTAAAGTGGAAGTAGAATATGTGTGGAT-3′
	Probe	5′-TTCTCAGAAACCCGATGGAGGCAATG-3′

Table 3.2 Nested PCR primer sequences

Region Amplified	Primer	Primer Sequence
HIV-1 JR-CSF 2562-3831	Fwd RT Outer	5'-GCTCTATTAGATACAGGAGC-3'
	Rev RT Outer	5'-CCTAATGCATATTGTGAGTCTG-3'
	Fwd RT Inner	5'-GTAGGACCTACACCTGTCAAC-3'
	Rev RT Inner	5'-CCTGCAAAGCTAGGTGAATTGC-3'
HIV-1 JR-CSF 4947-6372	Fwd vif Outer	5’-CAGGGACAGCAGAGATCC-3'
	Rev vif Outer	5’-GTGGGTACACAGGCATGTGTGG-3'
	Fwd vif Inner	5’-CTTTGGAAAAGGACCAGCAAAGC-3'
	Rev vif Inner	5’-GATGCACAAAATAGAGTGGTGG-3'
HIV-1 JR-CSF 8721-9441	Fwd nef Outer	5’-GAATAGTGCTGTTAGCTTGC-3'
	Rev nef Outer	5’-CAAAAAGCAGCTGCTTATATGCAGC-3'
	Fwd nef Inner	5’-TAGAGCTATTCGCCACATACC-3'
	Rev nef Inner	5’-CGGAAAGTCCCTTGTAGCAACG-3'

CHAPTER FOUR: Results

Introduction

Previous work has shown that HIV defective in Vif fails to replicate in human primary T cells and macrophages (Gabuzda, Lawrence et al. 1992; Gabuzda, Li et al. 1994). In the absence of Vif, APOBEC3G and 3F are encapsidated in progeny virions and deaminate cytosines in the viral DNA during reverse transcription, inducing G to A mutations in the coding strand of the viral genome (Harris, Bishop et al. 2003; Liddament, Brown et al. 2004; Suspene, Rusniok et al. 2006). This deamination, if unimpeded, destroys the virus and suppresses further propagation of HIV making the Vif/APOBEC axis a tantalizing therapeutic target. Recent *in vitro* work, however, has demonstrated that tissue culture adapted Vif-deficient HIV can in some instances persist in permissive cells transduced to express APOBEC3G. The adapted viruses only incur sub-lethal levels of APOBEC3G mutations (Hache, Shindo et al. 2008). These APOBEC3G resistant viruses, however, remained fully susceptible to restriction by APOBEC3F and were incapable of propagating in primary PBMC cultures and non-permissive T cell lines (Hache, Shindo et al. 2008).

In vitro, HIV that is defective in Vif has not been shown to overcome restriction by all APOBEC proteins; however, it has yet to be determined if APOBEC restriction of Vif-deficient HIV *in vivo* is sterilizing. The possibility exists that there are long-lived viral reservoirs and also that different cell populations can be infected *in vivo*. As HIV has evolved to encode a protein whose only identified function is to enable the virus to evade restriction by multiple APOBEC proteins, the loss of Vif would be expected to be deleterious to HIV *in vivo*. To address this, I have exposed humanized mice to HIV rendered defective in Vif, either by a deletion or by a one nucleotide insertion. My results show that in the absence of Vif, HIV replication in humanized mice is severely crippled, and analysis of viral DNA reveals extensive hypermutation by APOBEC. In some instances, however, HIV is able to restore Vif expression to protect itself from APOBEC restriction. Upon restoration of Vif, viral replication is unimpeded, demonstrating the strong *in vivo* selective pressure on HIV to maintain Vif.

In vitro analysis of Vif-deficient HIV-1_{JR-CSF}

HIV-1_{JR-CSF}, a CCR5 tropic primary HIV isolate from the cerebral spinal fluid of a patient, was chosen for these experiments as opposed to a tissue culture adapted virus CXCR4 tropic virus since CCR5 tropic HIV

is responsible for the majority of primary infections (Margolis and Shattock 2006). Vif-deficient HIV proviruses were generated by either a one nucleotide insertion or a large deletion in the vif open reading frame (ORF). The locations of these mutations were chosen such that they would not affect the overlapping *pol* or *vpr* genes (Figure 4.1a). Human embryonic kidney (HEK) 293T cells were chosen as the producer cell line because they lack expression of APOBEC proteins. Cells were transfected with either wild type HIV-$1_{JR\text{-}CSF,}$ HIV-$1_{JR\text{-}CSF}$vif(FS), or HIV-$1_{JR\text{-}CSF}$vif(Del) plasmid DNA, cells were harvested 48 hours post-transfection and whole cell lysates resolved by SDS-PAGE. Western blot analysis using a Vif polyclonal antiserum obtained from the NIH AIDS Research and Reference Reagent Program (Goncalves, Jallepalli et al. 1994) demonstrated the lack of expression following disruption of the *vif* ORF (Figure 4.1b).

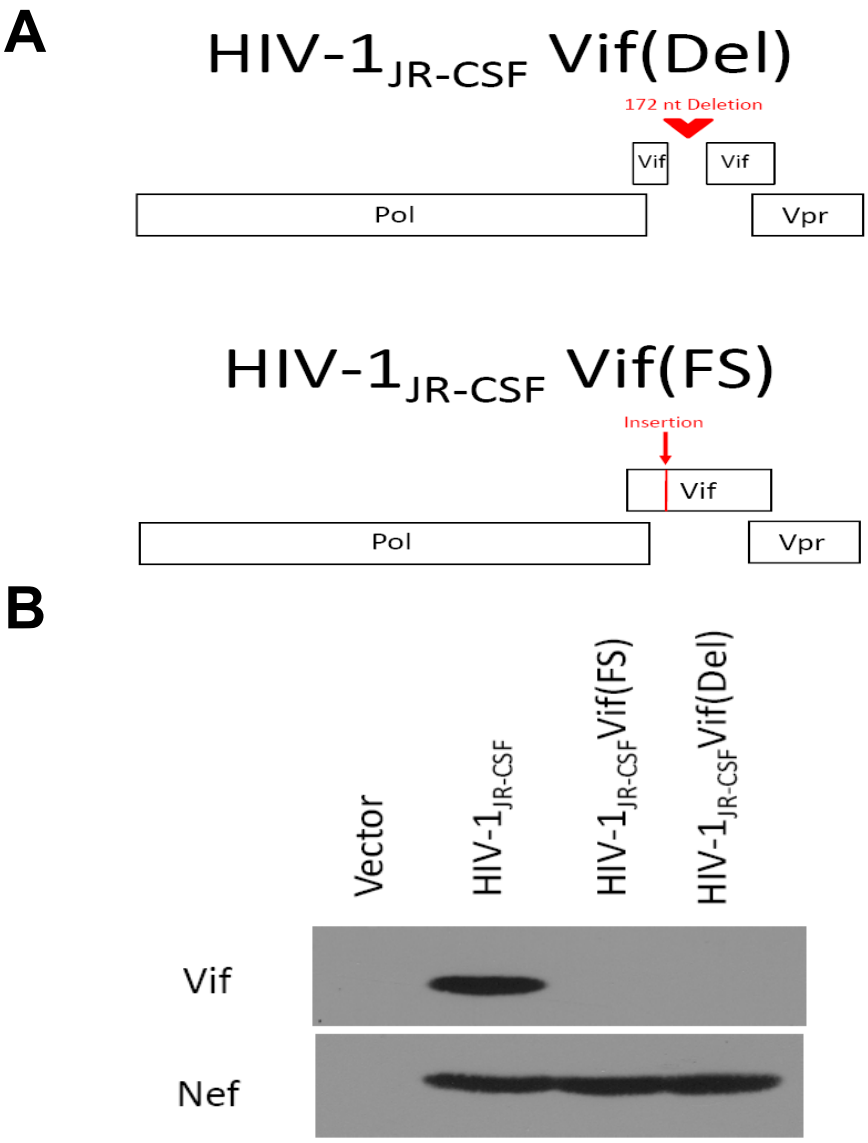


Figure 4.1 Mutations disrupting the *vif* open reading frame in HIV-1_{JR-CSF}.

(A) The location in the HIV-1 genome of either a 172 nucleotide deletion or a one base insertion to disrupt the *vif* gene. In both viruses the overlapping *pol* and *vpr* genes are not affected by the introduced mutations. (B) Western blot analysis of lysates from 293T cells transfected with either wild type or Vif-defective HIV-1 DNA for Vif expression. Expression of Nef was used as an internal control.

To confirm that the lack of Vif expression resulted in the inability of HIV to degrade APOBEC3G, wild type HIV-1_{JR-CSF}, HIV-1_{JR-CSF}vif(FS) or HIV-1_{JR-CSF}vif(Del) plasmid DNA was co-transfected into 293T cells with an expression vector for HA-tagged APOBEC3G obtained from the NIH AIDS Research and Reference Reagent Program (Sheehy, Gaddis et al. 2002; Stopak, de Noronha et al. 2003). Western blot analysis for APOBEC3G of whole cell lysates using an anti-HA antibody confirmed that only wild type HIV-1_{JR-CSF} is capable of degrading APOBEC3G (Figure 4.2). When APOBEC3G DNA was co-transfected with either HIV-1_{JR-CSF}vif(FS) or HIV-1_{JR-CSF}vif(Del), APOBEC3G was present in the lysates, demonstrating that the disruption of Vif prevents the degradation of APOBEC3G. Western blot analysis for Vif confirmed it is not expressed by HIV-1_{JR-CSF}vif(FS) or HIV-1_{JR-CSF}vif(Del). Western blot analysis for Nef (Arora, Molina et al. 2000; Foster, Molina et al. 2001) was performed as a control to confirm equal loading of viral proteins. Together these results demonstrate the disruption of the *vif* open reading frame and the resulting loss of Vif expression renders HIV unable to degrade APOBEC3G.

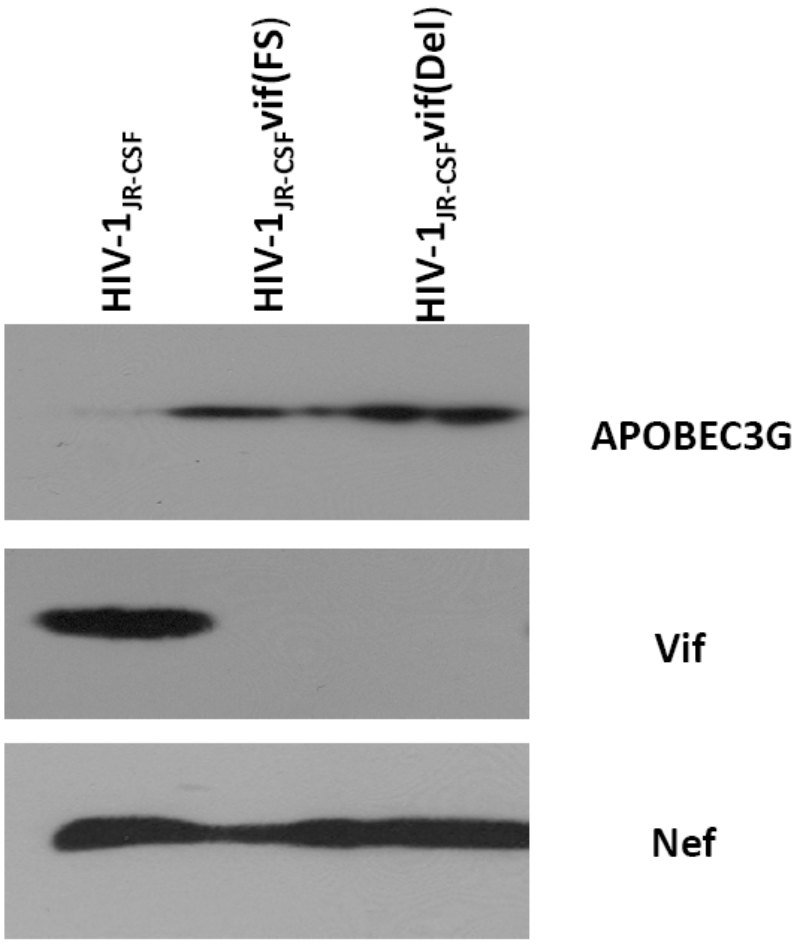
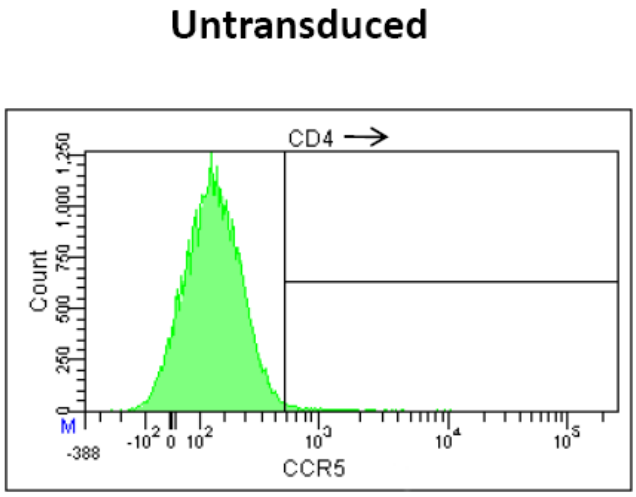


Figure 4.2 Vif-defective HIV-1 fails to degrade APOBEC3G.
Western blot analysis of lysates of 293T cells co-transfected with an APOBEC3G expression vector and either wild type (lane 1) or Vif-deficient HIV (lanes 2 & 3) demonstrating degradation of APOBEC3G only with HIV expressing Vif. Western blot analysis of Nef was used as a control for equal loading of viral proteins.

Vif-deficient HIV-1_{JR-CSF} replicates in a permissive cell line

To demonstrate that the two Vif-deficient HIV-1_{JR-CSF} viruses are replication competent, the propagation of the two mutant viruses in culture was compared to wild type HIV-1_{JR-CSF} in a permissive cell line. CEM-SS cells express CD4 and CXCR4 but do not express APOBEC3G or 3F and therefore permit replication of Vif-deficient HIV. However, they lack expression of CCR5 (CD195), the co-receptor required for infection by HIV-1_{JR-CSF}. Therefore, I had to create a CCR5 expressing permissive T cell line. To be able to compare the replication of Vif-deficient and wild type HIV-1_{JR-CSF}, CEM-SS cells were transduced with a retroviral vector containing CCR5. The transduced cells were selected in puromycin and sorted twice to obtain a $CD4^{Hi}CCR5^{Hi}$ population (Figure 4.3). Following the second sort for $CD4^{Hi}CCR5^{Hi}$ cells, the CEM-SS cells were maintained in puromycin selection and remained 95-97% $CCR5^{+}$.

A



B

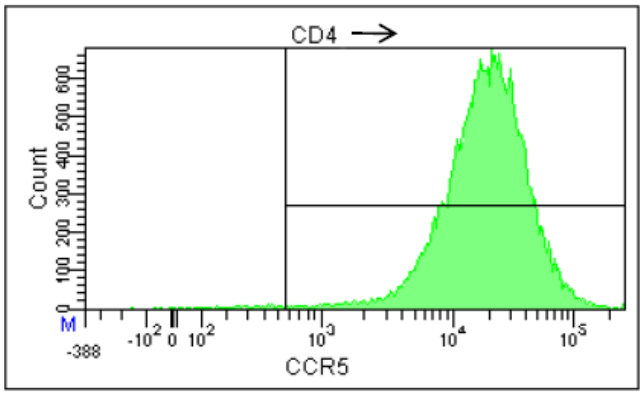


Figure 4.3 Cell surface expression of CCR5 on transduced CEM-SS cells.

Flow cytometric analysis of the human T cell line CEM-SS transduced to express the β-chemokine receptor CCR5 (CD195). Cells were analyzed for expression of both CD4 and CCR5. $CCR5^{+}CD4^{+}$ double positive cells are shown in (A) untransduced cells or (B) transduced.

CCR5 expressing CEM-SS cells were then infected with either Vif-deficient or wild type HIV-1_{JR-CSF} and the culture supernatant sampled at three day intervals to compare the propagation of the viruses by p24 analysis. Both Vif-deficient viruses replicated with kinetics similar to wild type HIV-1_{JR-CSF} in the permissive cell line (Figure 4.4), confirming that the loss of Vif by either mutation does not affect the replicative ability of HIV-1_{JR-CSF} in a permissive cell line. DNA from infected CCR5 expressing CEM-SS cells at day 3 and day 9 was isolated and the viral *nef* gene amplified and sequenced to demonstrate no G to A hypermutation characteristic of APOBEC occurring in the viral DNA sequences from the permissive cell line (Figure 4.5a).

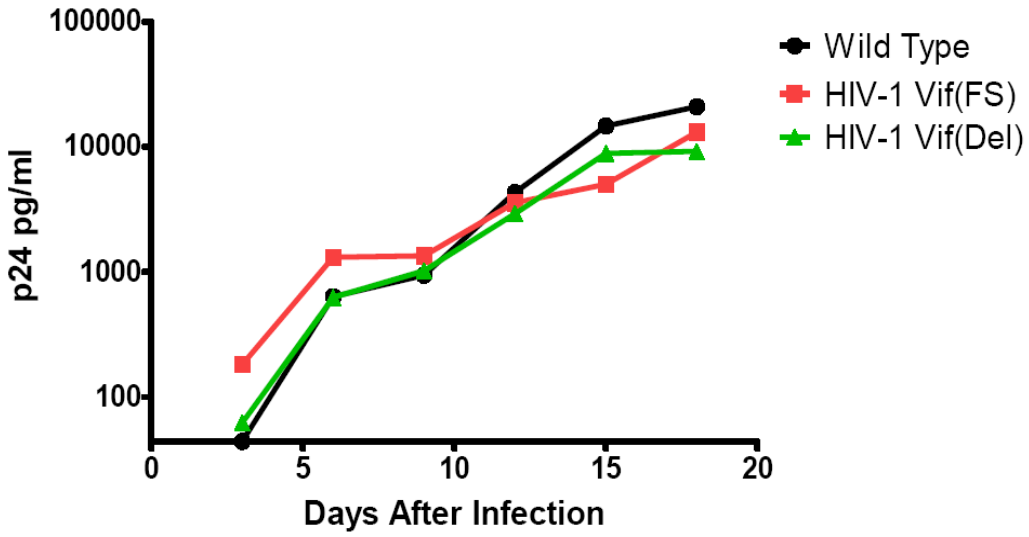


Figure 4.4 Wild type and Vif-defective HIV-1_{JR-CSF} replicate with similar kinetics in permissive cells.

CCR5 expressing CEM-SS cells infected with either wild type or Vif-deficient HIV. Propagation of HIV was monitored in the culture supernatants by p24 analysis, demonstrating equal replication of Vif expressing (black circles) and Vif-defective (red squares and green triangles) HIV in permissive cells.

As a control to demonstrate APOBEC3G mutagenesis, viruses generated by co-transfection of an APOBEC3G expression vector and viral DNA (wild type or Vif-defective) were used to infect CCR5 expressing CEM-SS cells. Viral DNA amplified and sequenced at three days post-inoculation showed no APOBEC3G mutations occurring in the wild type HIV provirus; however extensive G to A mutagenesis, as indicated by the green and dark blue lines was observed in the viral DNA from both Vif deleted and Vif frame shifted HIV (Figure 4.5b). The green lines indicate a G nucleotide mutated to A that is homogenous in the bulk DNA population, while the dark blue lines represent a G nucleotide that is a mixed population of either G or A at that position in the population sequenced as not every provirus present has been mutated at that specific nucleotide.

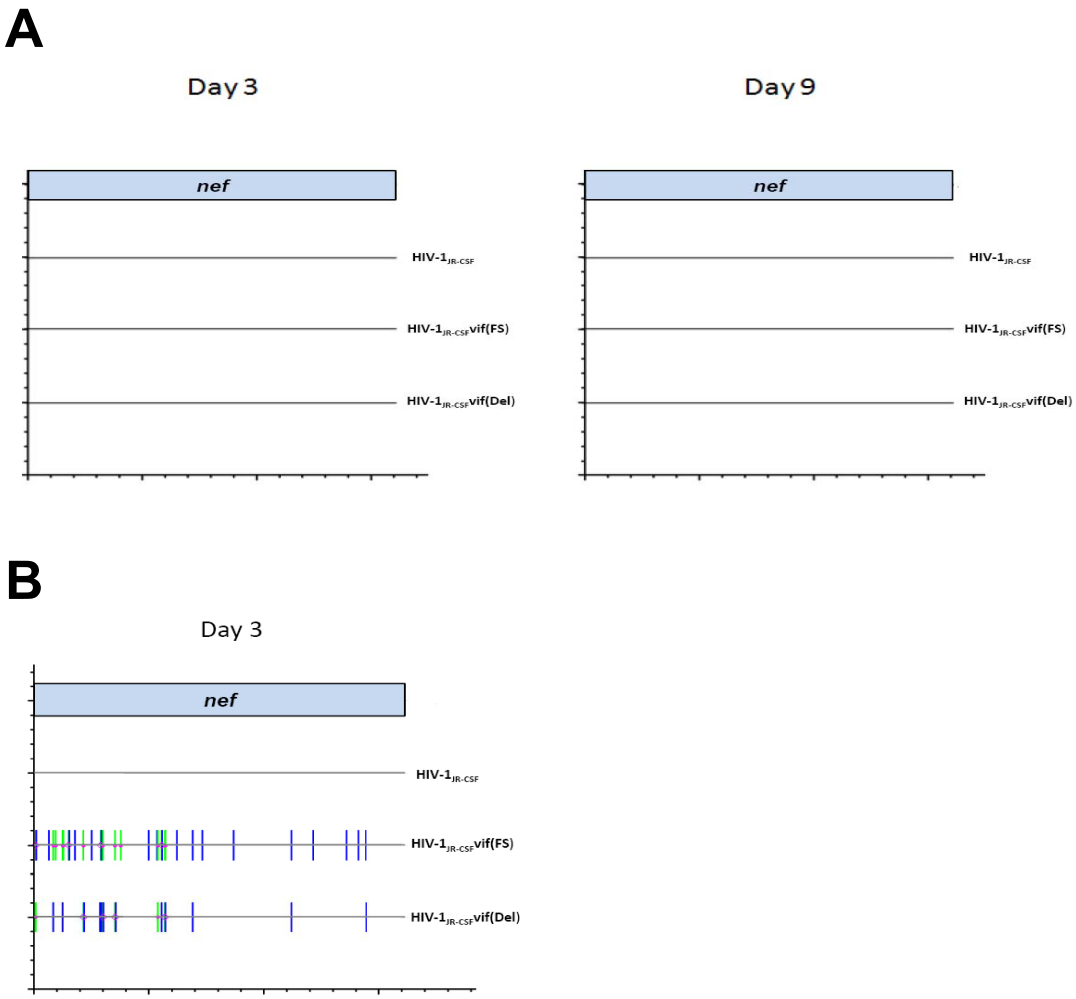


Figure 4.5 No G to A hypermutation of Vif-defective HIV-1_{JR-CSF} *in vitro* in the absence of APOBEC3G

The sequence of the *nef* gene from infected CEM-SS R5 was compared to the original proviral sequence (represented at the top in each sequence comparison) (A) Viral DNA from cells infected with wild type, Vif frame shift, or Vif deleted HIV at day 3 and day 9 showed no detectable G to A changes in the viral DNA sequence indicative of APOBEC3G mutagenesis. (B) Viral DNA from CCR5 expressing CEM-SS cells infected with viruses generated by co-transfection with APOBEC3G. Viral DNA from wild type HIV exhibits no detectable APOBEC mutations, however numerous G to A mutations are present in all (green lines) or a population (blue lines) of the *nef* sequences from cells infected with HIV-1_{JR-CSF}vif(FS) or HIV-1_{JR-CSF}vif(Del).

Human APOBEC3G and APOBEC3F mRNA is expressed in humanized mice

APOBEC3G and 3F are present in multiple human cell types. APOBEC mRNA expression in human tissues has been recently characterized (Koning, Newman et al. 2009). To evaluate the levels of APOBEC3G and 3F in different tissues from humanized mice, I used well characterized sets of primers to amplify APOBEC3G or 3F mRNA (Vetter, Johnson et al. 2009). Their specificity was fully confirmed using mRNA from lysates from 293T cells transfected with human APOBEC3B, 3G, or 3F. To verify expression of human APOBEC3G and 3F in humanized mice, RNA levels were measured by quantitative real-time PCR (qRT-PCR) from cells isolated from the spleen, bone marrow, mesenteric lymph nodes, liver, and lung. To determine absolute copy numbers of mRNA, human APOBEC3G or 3F RNA was in vitro transcribed from an expression vector, quantified, and serially diluted to generate a standard curve. As a negative control, RNAs from cells isolated from tissues of a non-humanized mouse were used. Both human APOBEC3G and 3F RNA were present in all tissues analyzed from the humanized mouse (Figure 4.6) demonstrating the ubiquitous expression of APOBEC3G or 3F mRNA in humanized mice.

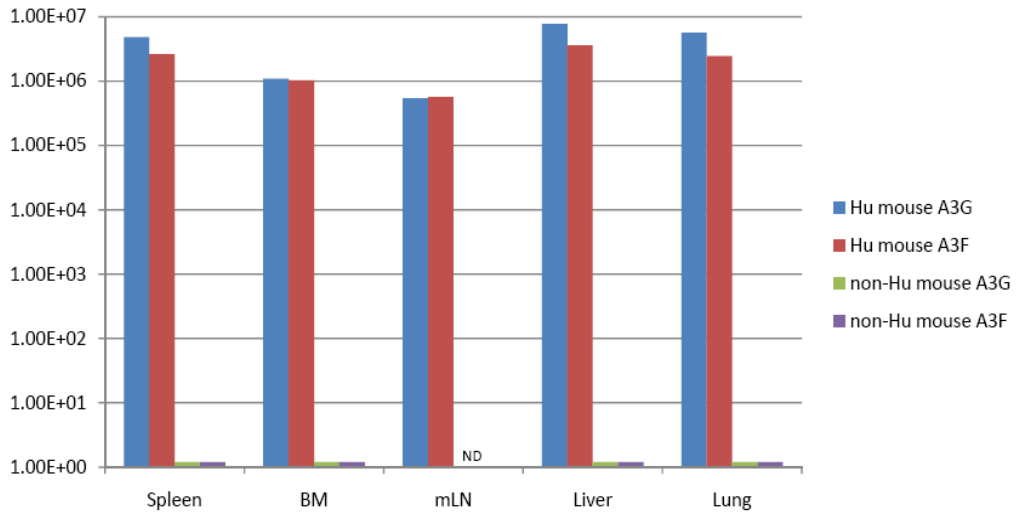


Figure 4.6 Ubiquitous APOBEC3G and 3F expression in humanized mice.

Quantitative real-time PCR analysis of human APOBEC3G (blue) and human APOBEC3F (red) mRNA from the tissues of a humanized mouse. To rule out cross reactivity of the primers with murine APOBEC3, mRNA from the tissues of a non-humanized mouse were also analyzed using the same primers for human APOBEC3G (green) and APOBEC3F (purple).

The primers for both human APOBEC3G and 3F did not amplify RNA purified from cells from any tissue of non-humanized mice, confirming that the primers do not cross-react with murine APOBEC3 RNA. These results confirm expression of both APOBEC3G and 3F mRNA in cells isolated from all the tissues analyzed from humanized mice.

Vif-deficient HIV-1_{JR-CSF} establishes infection *in vivo*

After confirming that both vif-defective viruses replicate comparably to wild type HIV-1_{JR-CSF} in a permissive cell line, humanized mice were intravenously exposed to $3.6x10^5$ tissue culture infectious units (TCIU) of HIV-1_{JR-CSF}vif(Del) or either $3.6x10^5$ or $9x10^4$ TCIU of wild type HIV-1_{JR-CSF} or HIV-1_{JR-CSF}vif(FS). To determine if Vif-defective HIV can establish infection *in vivo*, mice were sacrificed 1 week after innoculation and the tissues harvested and analyzed for the presence of viral DNA. The results summarized in table 4.1 show that the two mice receiving wild type HIV-1_{JR-CSF} at either the high dose ($3.6x10^5$ TCIU) or the low dose ($9x10^4$ TCIU) have viral DNA present in every tissue analyzed, demonstrating a systemic distribution and active replication of the virus.

Virus	Mouse	Tissue						
		Peripheral Blood	Spleen	Bone Marrow	Lymph Nodes	Thymus	Liver	Lung
Wild Type HIV-1_{JR-CSF}	W1	+	+	+	+	+	+	+
	W2	+	+	+	+	N/A*	+	+
HIV-1_{JRCSF} vif(Del)	Del1	-	-	-	-	-	-	+
	Del2	-	-	-	-	-	+	-
	Del3	-	-	-	-	-	+	-
HIV-1_{JRCSF} vif(FS)	FS1	-	+	-	+	-	-	+
	FS2	-	+	-	-	-	-	-
	FS3	-	-	-	-	-	-	+
	FS4	-	+	-	+	-	-	-

* Not anlayzed

Table 4.1 Vif-defective HIV establishes infection in humanized mice. Humanized mice injected intravenously with wild type or Vif-deficient (deleted or frame shifted) HIV. The mice were analyzed for evidence of systemic infection at one week post-exposure by nested PCR of the viral *nef* gene to detect viral DNA in the tissues. Tissues positive for viral DNA are denoted by +, negative tissues indicated by -.

In the seven mice receiving the Vif-deficient viruses, the replication of these viruses in the absence of Vif was severely crippled as viral DNA was found in only in a few tissues in each mouse, specifically the spleen, lymph nodes, liver, and lung as opposed to the systemic distribution seen with wild type HIV-1_{JR-CSF} (Table 4.1).

Viral DNA amplified from tissues obtained from infected humanized mice was sequenced and compared to the original proviral sequence to determine if G to A hypermutation characteristic of APOBEC was occurring in the absence of Vif. The viral *nef* sequences amplified from tissues of the two humanized mice infected with wild type HIV-1_{JR-CSF} at 1 week post-exposure had no G to A changes (Figure 4.7a), demonstrating that in the presence of Vif, the viral DNA was not subjected to APOBEC mutagenesis. The *nef* sequences amplified from the viral DNA from three humanized mice infected with HIV-1_{JR-CSF}vif(Del) showed up to 26 G to A mutations (Figure 4.7b), all at putative APOBEC target sites (shown as green lines with pink dots).

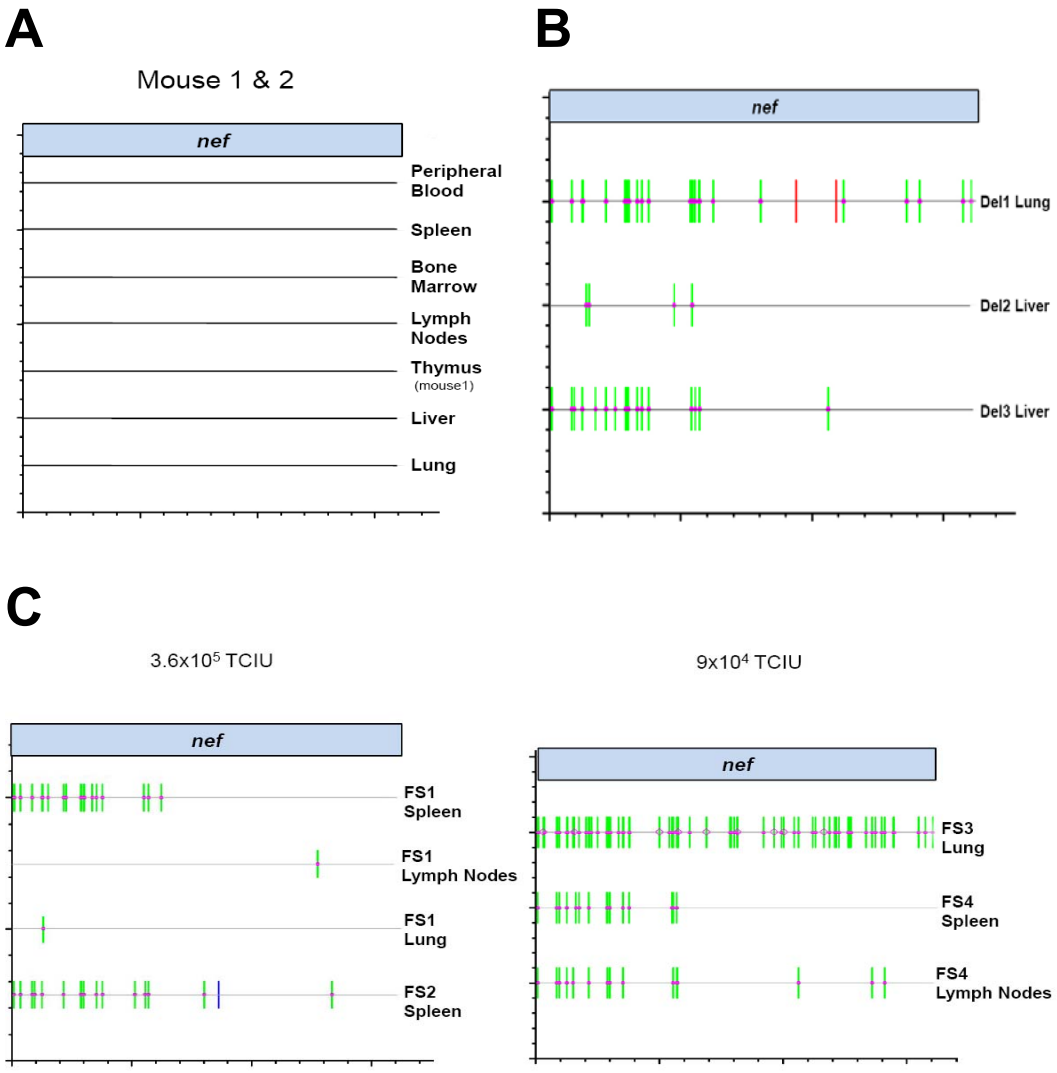


Figure 4.7 APOBEC hypermutation of Vif-deficient HIV-1 provirus at one week post exposure *in vivo*.

Viral *nef* sequences at one week post-exposure from (A) combined data from two mice receiving wild type HIV-1_{JR-CSF}, (B) three mice (Del1-3) receiving Vif-deleted HIV-1_{JR-CSF}, or (C) four mice receiving Vif frame-shifted HIV-1_{JR-CSF} (FS1-4) at either $3.6x10^5$ or $9x10^4$ TCIU compared to the original proviral sequence (top of each alignment). Wild type HIV DNA incurred no G to A mutations, however, DNA from Vif-defective HIV was mutated by APOBEC in humanized mice.

In addition to the G to A mutations, the *nef* sequence from Del1 had two C to T changes (red lines), indicative of APOBEC3G deamination of cytosines occurring on the plus strand of the viral DNA. These two plus strand mutations both occurred downstream of the 3'PPT, a region of the plus strand DNA that is transiently single stranded during reverse transcription making it a potential substrate for APOBEC. Viral DNA sequences analyzed from the four humanized mice receiving $3.6x10^5$ TCIU or at $9x10^4$ TCIU of HIV-$1_{JR\text{-}CSF}$vif(FS) also had G to A mutations present in every sequence analyzed, indicating wide spread APOBEC mutagenesis occurring in the absence of a functional Vif (Figure 4.7c). Interestingly, variable levels of G to A mutations in the viral DNA from these mice were observed, both between mice and also between tissues from the same mouse. Viral DNA from FS1 receiving $3.6x10^5$ TCIU of virus had 17 mutations in the proviral sequence isolated from the spleen, but only 1 G to A mutation in viral DNA from each of the lung and lymph nodes, indicating different populations of viral DNA are present in the different tissues of this mouse. In the two mice receiving $9x10^4$ TCIU of virus, FS3 had 69 G to A mutations in the viral DNA isolated from the lung, while mouse FS4 had 14 and 15 mutations in viral DNA from the spleen and lymph nodes respectively, showing variable levels of APOBEC mutagenesis occurring between mice. These results confirm that HIV-$1_{JR\text{-}}$

$_{CSF}$ defective in Vif, either with a deletion or a one base insertion can infect humanized mice through an intravenous exposure. While Vif defective HIV-1 does establish infection in 100% of mice, replication of these viruses is severely crippled compared to wild type HIV-1 in humanized mice.

Wild type HIV-1$_{JR-CSF}$ is not subjected to APOBEC restriction in humanized mice

After confirming that intravenous inoculation with Vif deficient HIV-1$_{JR-CSF}$ results in infection in humanized mice, I determined the course of infection that occurs with wild type HIV-1$_{JR-CSF}$ over an extended period of time, up to 12 weeks to allow comparison to long term infection with Vif defective HIV. Humanized mice were exposed intravenously to $3x10^5$ TCIU of HIV-1$_{JR-CSF}$ and the peripheral blood monitored for $CD4^+$ T cell levels as well as for viral antigenemia. Viral replication is observed in the periphery as early as two weeks post exposure as measured by plasma antigenemia (Figure 4.8a). The peak of antigenemia is accompanied by a substantial loss of $CD4^+$ T cells in the peripheral blood, which decline by approximately 50% and do not return to pre-exposure levels during the course of the infection. Viral DNA amplified from peripheral blood cells

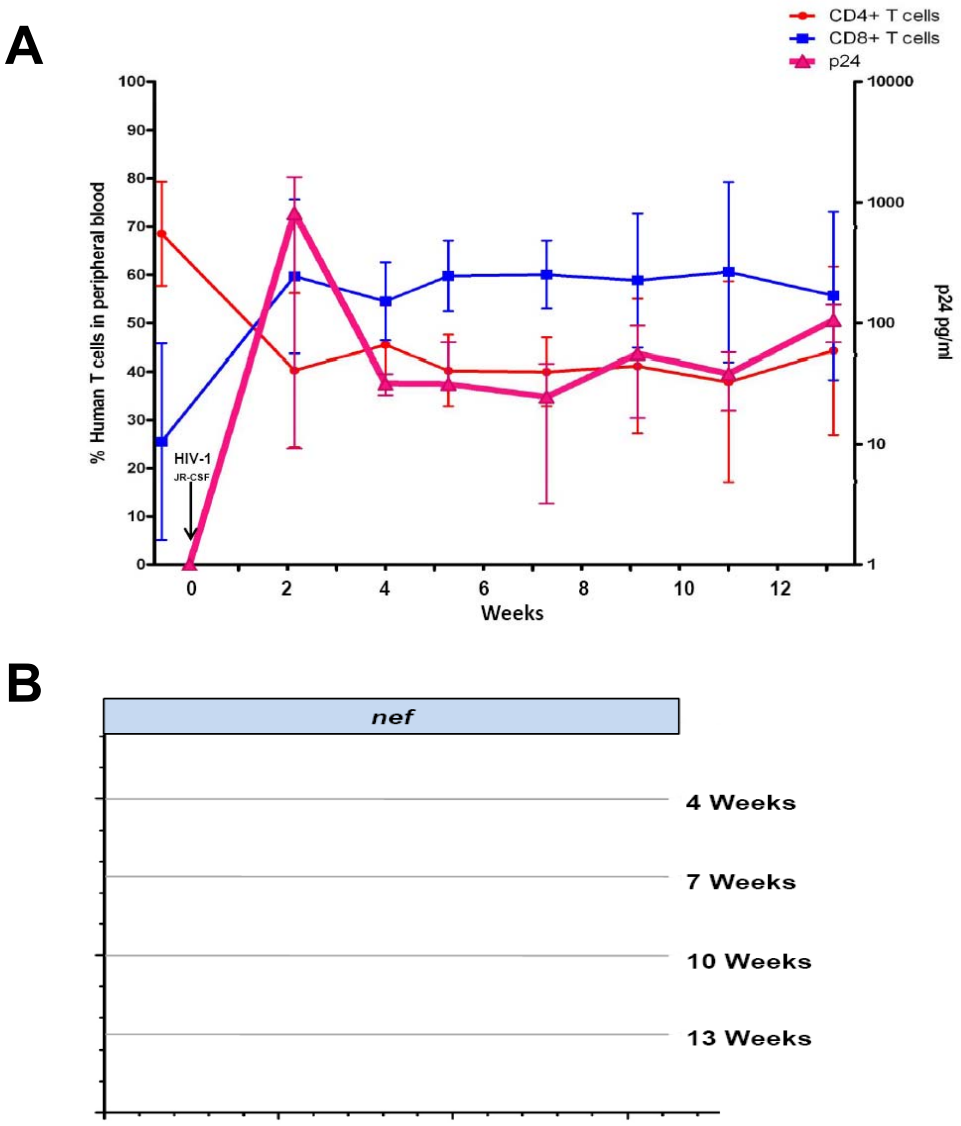


Figure 4.8 Wild type HIV-1_{JR-CSF} is not subjected to APOBEC mutagenesis in humanized mice.

(A) Analysis of peripheral blood from three mice infected intravenously with HIV-1 for $CD4^+$ and $CD8^+$ T cells and viral antigenemia through 13 weeks post infection. Viral breakthrough is observed at two weeks and is accompanied by a loss in $CD4^+$ T cells. (B) Comparison of *nef* sequences from viral DNA in peripheral blood cells of a wild type HIV exposed mouse at multiple time points during infection against the original proviral sequence showed no detectable APOBEC hypermutation.

from humanized mice at multiple time points throughout the course of infection showed no detectable G to A mutations at any time point, further demonstrating that wild type HIV-1_{JR-CSF} is not subjected to APOBEC mutagenesis (Figure 4.8b).

Vif deleted HIV-1_{JR-CSF} is lethally restricted by APOBEC *in vivo*

Based on the ability of Vif defective HIV to adapt *in vitro* to evade lethal restriction by APOBEC3G (Hache, Shindo et al. 2008), I hypothesized that Vif deleted HIV-1_{JR-CSF} would persist and ultimately escape *in vivo* APOBEC restriction through compensatory second site mutations. To test this hypothesis, I inoculated five mice intravenously with $3.6x10^5$ TCIU of HIV-1_{JR-CSF} vif(Del) and analyzed the peripheral blood at regular intervals for evidence of plasma antigenemia. We also monitored the levels of peripheral blood $CD4^+$ T cells by flow cytometry. There were no significant changes observed in the levels of $CD4^+$ T cells throughout the experiment and in all of these mice no viral antigenemia was detected in plasma at any time point analyzed (Figure 4.9), demonstrating a lack of virus replication in the periphery of the humanized mice.

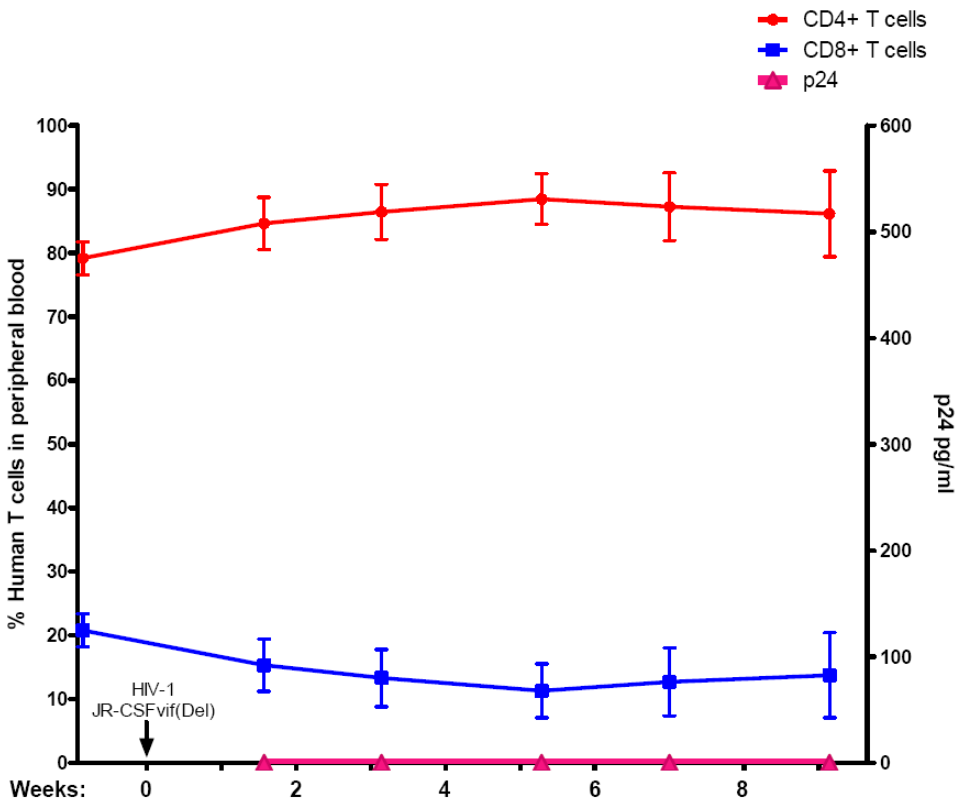


Figure 4.9 Failure of Vif-deleted HIV-$1_{JR\text{-}CSF}$ to replicate in peripheral blood.

Analysis of human $CD4^+$ and $CD8^+$ T cells and viral antigenemia in the peripheral blood of five humanized mice exposed intravenously to HIV-$1_{JR\text{-}CSF}$Vif(Del) shows no evidence of viral replication and no loss of $CD4^+$ T cells through nine weeks post-exposure.

Virus	Dose	Mouse	Viral Load (HIV-1 RNA copies/ml plasma)			
			Week 2/3	Week 4/5	Week 6/7	Week 9/10
$HIV\text{-}1_{JR\text{-}CSF}$ Vif (FS)	$3.6x10^5$	FS5	<400	<400	<400	<400
		FS6	<400	<400	<400	<400
		FS7	<400	<400	<400	<400
		FS8	<400	<400	<400	<400
		FS9	<400	<400	<400	<400
		FS10	<400	ND	<400	
		FS11	$1.04x10^5$	$2.40x10^5$	$1.58x10^5$	$2.97x10^5$
		FS12	$8.27x10^4$	$1.31x10^5$	$2.14x10^5$	
		FS13	<400	<400	$4.96x10^3$	$8.65x10^4$
	$9x10^4$	FS14	$2.91x10^5$	$8.34x10^5$	$4.90x10^5$	
		FS15	<400	<400	<400	<400
		FS16	<400	<400	<400	<400
		FS17	<400	<400	<400	<400
		FS18	<400	<400	<400	<400
$HIV\text{-}1_{JR\text{-}CSF}$ Vif(Del)	$3.6x10^5$	Del4	<400	<400	<400	<400
		Del5	<400	<400	<400	<400
		Del6	<400	<400	<400	
		Del7	<400	<400	<400	<400
		Del8	<400	<400	<400	<400

ND= Not Done

Table 4.2 Viremia detected in only 4 of 19 mice receiving Vif-defective HIV-1.
Quantitation of viral RNA in the plasma from mice receiving frame shifted or deleted Vif $HIV\text{-}1_{JR\text{-}CSF}$ at the listed dose. Viral RNA was detected in only four mice, listed as the copies of RNA/ ml of plasma, indicating viral replication occurred in the periphery of these mice. In all other mice, viral RNA was not detected at any time points analyzed. Shaded boxes indicate the experiment was not carried out to the designated time point.

Additionally, plasma from all mice was analyzed for the presence of viral RNA using a sensitive RT-PCR viral load assay. All mice receiving Vif-deleted HIV-1_{JR-CSF} were negative for viral RNA at every time point analyzed (Table 4.2). Cells from peripheral blood were analyzed for the presence of viral DNA by PCR using two different sets of nested PCR primers, however, no cell associated viral DNA was detected at any time point. These results are in sharp contrast to those obtained with wild type HIV-1 and demonstrate a lack of viral replication in the periphery the absence of Vif.

I then analyzed these mice to determine if there was an occult infection, showing no evidence in the periphery, yet present in other tissues. For this purpose I determined the presence of proviral sequences in tissues from the HIV-1_{JR-CSF} vif(Del) injected humanized mice. Mice were harvested at 6 and at 8 weeks post-exposure. Tissues from the remaining three humanized mice were collected 10 weeks post-exposure. Cells isolated from these tissues were analyzed for viral DNA using at least two different sets of nested PCR primers. One of the mice analyzed at 10 weeks as well as the mouse analyzed at 6 weeks post exposure were negative for viral DNA in all tissues analyzed. Viral DNA was

detected in the lungs from two mice, Del5 and Del8, with primers to reverse transcriptase and in the lung and lymph node from the mouse harvested at 8 weeks, Del7, with primers for the *nef* gene. The limited presence of viral DNA in lymphoid tissues from mice infected with vif-deleted HIV as opposed to the systemic distribution observed with wild type HIV infection suggests that the Vif defective virus is unable to overcome APOBEC restriction.

Extensive APOBEC hypermutation of viral DNA in the absence of Vif

Sequence analysis of the amplified viral DNA from the lung and the lymph nodes from Del4 analyzed at 8 weeks post exposure revealed 18 G to A mutations in viral DNA from each tissue in the absence of Vif (Figure 4.10a). Interestingly, the viral DNA sequences amplified from the lung and the lymph nodes are identical, showing the exact same pattern of G to A hypermutation. Two of these G to A mutations disrupt the *nef* reading frame, as one changes the initiation codon to an isoleucine (red asterisk) and a second change results in a tryptophan to stop codon mutation (black asterisk).

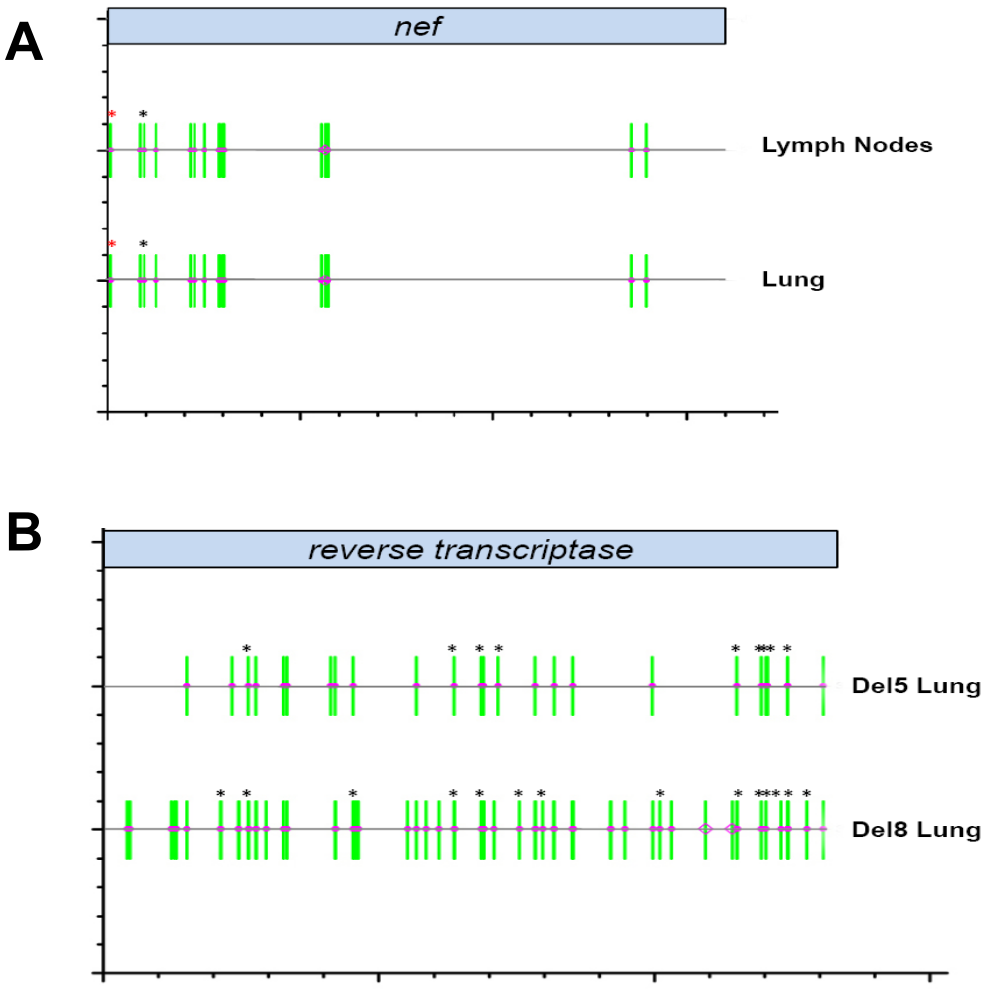


Figure 4.10 Viral DNA recovered from humanized mice infected with Vif-deleted HIV-1_{JR-CSF} is extensively mutated by APOBEC.

(A) Sequence comparison of *nef* amplified from lymph node and lung of mouse Del4 exposed to HIV-1_{JR-CSF} Vif(Del) shows the same pattern of G to A mutations at 8 weeks post exposure with two mutations disrupting the reading frame (asterisks). (B) Sequence comparison of reverse transcriptase region of the *pol* gene amplified from the lung of mouse Del5 or Del8 at 10 weeks post infection shows APOBEC hypermutation, with numerous mutations introducing premature stop codons (asterisks). The top of each alignment represents the original gene sequence from the virus, lower lines represent the viral DNA sequence recovered from the indicated tissue and mouse.

Surprisingly, one G to A mutation was at the dinucleotide sequence GT, not at a putative APOBEC restriction site (green line with pink open diamond) and potentially represents a reverse transcriptase error.

The two mice analyzed at 10 weeks post inoculation both had viral DNA detected only in the cells from the lung. Sequence analysis revealed extensive G to A hypermutation (Figure 4.10b) of the provirus in the Del5 mouse. In this case 25 APOBEC induced mutations 9 of which resulted in tryptophan codons being changed to stop codons disrupting the reverse transcriptase gene (black asterisks). The amplified viral DNA from the Del8 mouse had 60 G to A mutations, 14 of which represent tryptophan to stop codons changes (black asterisks). Of the 60 G to A mutations, two are at the dinucleotide sequence GT with the G mutated to A, which is not a putative APOBEC3G or APOBEC3F restriction site (green line with pink open diamond), again suggesting a reverse transcription error.

In the three mice where viral DNA was detected, the sequences were all extensively mutated by APOBEC, demonstrating in vivo restriction of HIV. In all viral sequences, G to A changes were found to disrupt essential viral genes, either with tryptophan codons mutated to stop codons or by disruption of the initiation codon by a methionine to

isoleucine change, further demonstrating the deleterious effect of APOBEC on HIV. The observation that viral DNA could be detected in cells from the lung in all three mice where HIV-1 DNA was present suggests that either low levels of viral replication were able to occur in the lung in the absence of Vif, or that possibly long-lived cells harboring viral DNA existed in the lungs of the humanized mice for an extended period of time to allow the detection of viral DNA up to 10 weeks post exposure.

In five mice exposed to Vif-deleted HIV-1JR-CSF, no viral breakthrough was observed up to nine weeks post-inoculation. Analysis of the peripheral blood of all mice showed no loss of $CD4^+$ T cells no detectable viral DNA at any time point analyzed. No viral DNA was found in any tissues from two of the mice. In the three mice where viral DNA was found in tissues, extensive G to A hypermutation characteristic of APOBEC was observed, demonstrating lethal restriction of Vif-deficient HIV *in vivo*.

Humanized mice infected with HIV-1_{JR-CSF} vif(FS)

After establishing that HIV-1_{JR-CSF} with a non-revertible deletion disrupting Vif is lethally restricted by APOBEC *in vivo*, I wished to evaluate

the selective pressure exercised on the virus by APOBEC to determine if HIV would be able to restore Vif to survive. To address this, nine humanized mice were exposed intravenously to $3.6x10^5$ TCIU of $HIV\text{-}1_{JR\text{-}CSF}$ vif(FS) and peripheral blood was monitored for evidence of viral replication by p24 ELISA for plasma antigenemia, nested PCR for the presence of cell associated viral DNA, and flow cytometric analysis for loss of $CD4^+$ T cells. Cells isolated from different tissues were also harvested and analyzed for viral DNA by PCR using nested primers that amplify two different regions of the viral genome. Six of the exposed humanized mice, FS5, FS6, FS7, FS8, FS9, and FS10 showed no plasma antigenemia and no loss of $CD4^+$ T cells in the peripheral blood at any time point analyzed (Figure 4.11). Additionally, these six mice were found to be negative for viral RNA in the plasma at all time points analyzed (Table 4.2). Nested PCR analysis detected no viral DNA in the peripheral blood of 4 of these mice at any time points analyzed.

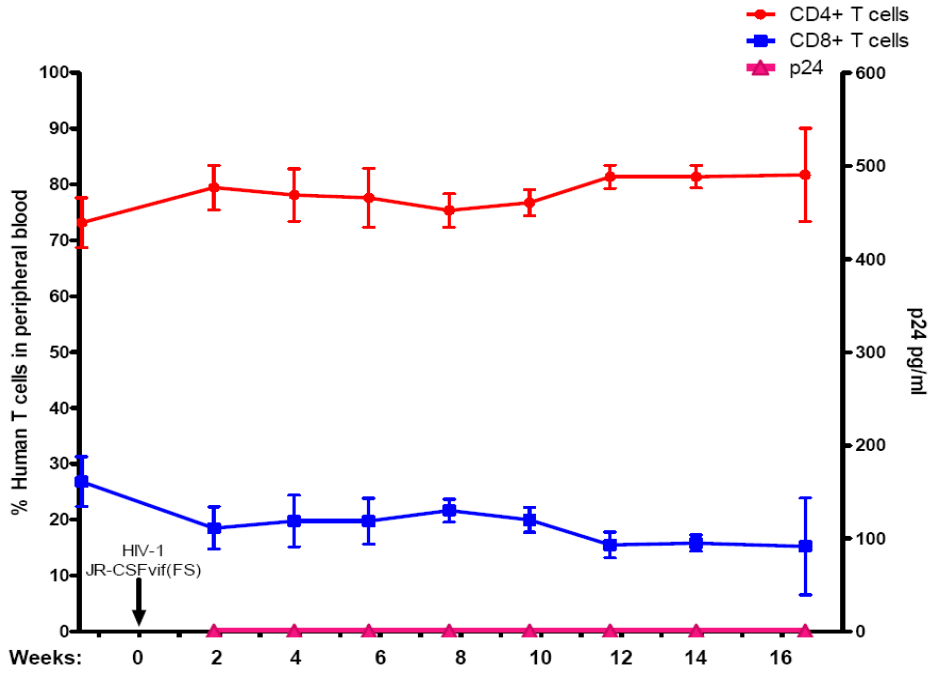


Figure 4.11 Mice exposed to HIV-$1_{JR\text{-}CS}$Vif(FS) show no evidence of viral replication and no loss of $CD4^+$ T cells in the peripheral blood.

Peripheral blood of 6 humanized mice exposed intravenously to $3.6x10^5$ TCIU of HIV-$1_{JR\text{-}CSF}$vif(FS) was monitored for loss of $CD4^+$ T cells and viral antigenemia through 16 weeks post exposure. In all 6 mice, no viral replication as measured by p24 or loss of CD4+ T cells was observed at any time point.

The tissues from three of these mice were collected, FS10 at 8 weeks and both FS7 and FS8 at 16 weeks post infection, and analyzed for viral DNA by nested PCR for *nef*, RT, and 1.4 kb region from nucleotides 4947-6372, which includes *vif, vpr* and *vpu*. Viral DNA was detected in two of these mice, the mouse analyzed at 8 weeks, FS10, had viral DNA in the lung with primers to *nef* and *vif*. In the lymph nodes viral DNA was amplified by primers for *vif.* One of the mice analyzed at 16 weeks, FS8, had viral DNA only in lymph nodes and this provirus was detected only with primers to RT. Extensive attempts to amplify other viral genes in tissues found to be positive with another primer set were unsuccessful. The tissues from FS7 showed no viral DNA by any of the three nested PCR primer sets.

Analysis of the viral DNA sequences from these mice showed extensive APOBEC mutagenesis. The viral DNA from the cells isolated from the lung of the FS10 was heavily mutated by APOBEC in two different regions of the viral genome. The *nef* gene had 46 G to A mutations, with one disrupting the initiation codon and three mutations changing tryptophan residues to stop codons (Figure 4.12a). The 1.4 kb

region from nucleotides 4947-6372, which includes *vif, vpr* and *vpu*, as well as part of the *pol*, *tat, rev*, and *env* genes, had 22 G to A mutations, with mutations that disrupted both the *tat* and *vpu* open reading frames (Figure 4.12b). This same region of viral DNA amplified from cells isolated from the lymph nodes had 46 G to A mutations with tryptophan to stop mutations disrupting *vpu, tat,* and *vpr* (black asterisks). Additionally, the initiation codons of *vif, vpr, tat*, and *env* were all disrupted by G to A mutations changing the methionine to isoleucine (red asterisks).

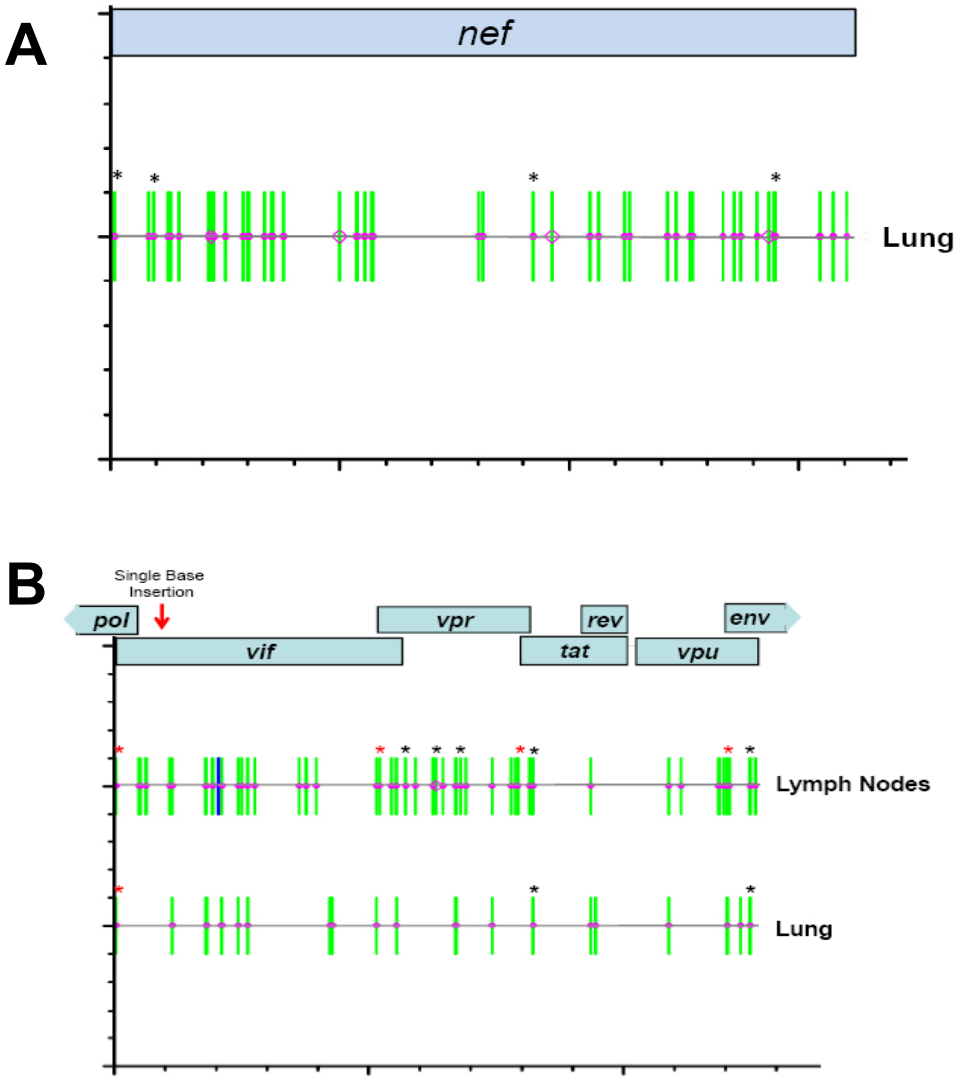


Figure 4.12 Extensive APOBEC mutagenesis in two regions of the viral DNA from mouse FS10.

Comparison of viral DNA amplified from lymph node and lung of mouse FS10 to the original HIV-$1_{JR\text{-}CSF}$Vif(FS) provirus sequence shows APOBEC hypermutation at both the 3' end of the viral genome (A) and in a 1.4kb region from nucleotides 4947-6372 (B) with mutations disrupting initiation codons (red asterisks) and changing tryptophan codons to stop codons (black asterisks). The top of each panel represents the original gene sequence from the virus, lower lines represent the viral DNA sequence recovered from the indicated tissue.

Viral DNA amplified from cells isolated from the lymph nodes of the mouse analyzed at 16 weeks, FS8, post exposure also was heavily mutated by APOBEC (Figure 4.13). The reverse transcriptase region had 49 G to A mutations, resulting in 13 tryptophan codons being changed to stop codons. Two of the 49 G to A changes are not at canonical APOBEC sites, and may represent reverse transcription errors. The viral DNA sequences analyzed from all of these mice were extensively mutated by APOBEC, with G to A hypermutation disrupting several viral genes, demonstrating lethal restriction of the virus by APOBEC. The absence of both plasma antigenemia and viral DNA in peripheral blood at all time points suggests the lethal restriction occurred early in infection, as no evidence of viral replication was detected in these mice.

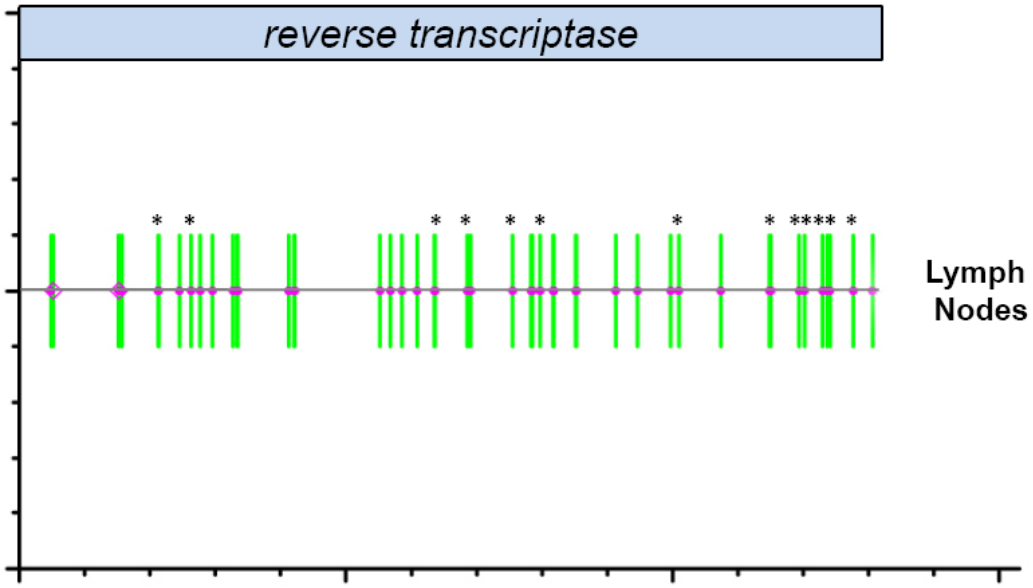


Figure 4.13 Hypermutation of RT in viral DNA recovered from FS8 demonstrates lethal restriction by APOBEC.

The box at the top of the figure represents the gene sequence from the original provirus. The lower line illustrating the sequence of viral DNA amplified from the lymph node of mouse FS8 at 16 weeks post exposure showed 49 G to A mutations in RT of *pol* gene. In this 1.2kb region, 13 tryptophan codons have been mutated to stop codons, disrupting the open reading frame (black asterisks).

Viral replication occurring early post exposure

Two mice exposed to HIV-1_{JR-CSF}(FS) showed evidence of virus replication at early time points post exposure. One mouse, FS6, had detectable viral DNA in peripheral blood cells at two weeks post infection. Sequence analysis of the amplified *nef* gene showed 17 APOBEC mutations, with one disrupting the initiation codon and three G to A mutations changing tryptophans to stop codons (Figure 4.14a). Viral DNA was not detected in peripheral blood of this mouse at any other time point through 16 weeks; however viral DNA was found in the lung of this mouse after harvest. Viral DNA was not detected in any other tissue analyzed. Surprisingly, the viral DNA sequence of the *nef* gene from the lung at 16 weeks post infection was identical to the sequence found 2 weeks post infection in the peripheral blood with the exact same pattern of APOBEC hypermutation. The reverse transcriptase region of the viral *pol* gene was amplified and sequenced from the lung of this mouse, revealing 22 APOBEC mutations including 9 G to A mutations changing tryptophan codons to stop codons (Figure4.14b). The viral sequences from this mouse highlight the deleterious effects of APOBEC hypermutation on HIV, with G to A changes disrupting viral genes essential for replication.

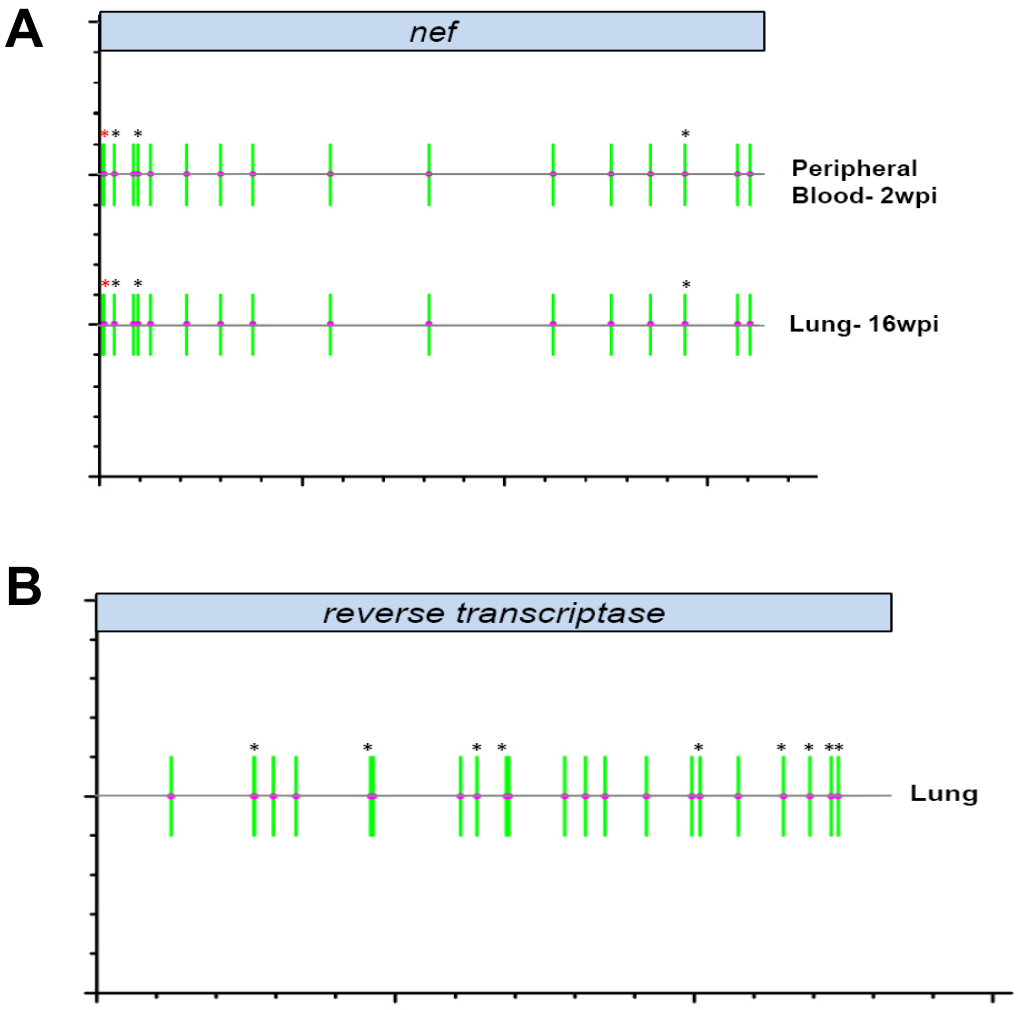


Figure 4.14 Hypermutated viral DNA detected in peripheral blood at 2 weeks and at 16 weeks in the lung.

(A) Nef sequences from peripheral blood at 2 weeks post infection and from the lung at 16 weeks post infection compared to the original provirus sequence showed the same 17 G to A mutations in both recovered sequences. The nef ORF is disrupted by 3 tryptophans changed to stop codons (black asterisks) and the initiation codon mutated to isoleucine (red asterisks). (B) Comparison of the sequence of RT from the lung to the original provirus showed 22 G to A mutations, 9 of which introduce stop codons (black asterisks), demonstrating lethal restriction by APOBEC.

The second mouse, FS9, exposed to HIV-1_{JR-CSF} vif(FS) with viral DNA in peripheral blood 4 and 6 weeks post exposure, did not have it at any other time point analyzed. The viral *nef* gene was amplified and sequenced and revealed 18 and 12 G to A mutations at 4 and 6 weeks respectively. At both time points an APOBEC mutation was disrupting the initiation codon of *nef* (red asterisks), however, the locations of some of the other mutations were different between the two time points, suggesting that at least two different proviral populations were present in the peripheral blood cells of this humanized mouse (Figure 4.15a). Additionally, a G to A mutation not at a putative APOBEC site (GGC to AGC) introduced a termination codon (black asterisk) in Nef at 4 weeks. Nested PCR analysis of cells isolated from different tissues showed evidence of viral DNA in the spleen, bone marrow and liver. Sequences of the *nef* gene from the spleen and the bone marrow showed 21 and 22 G to A mutations respectively and viral sequences from both tissues had the initiation codon disrupted (red asterisks) as well as tryptophans changed to stop codons (black asterisks) (Figure 4.15b).

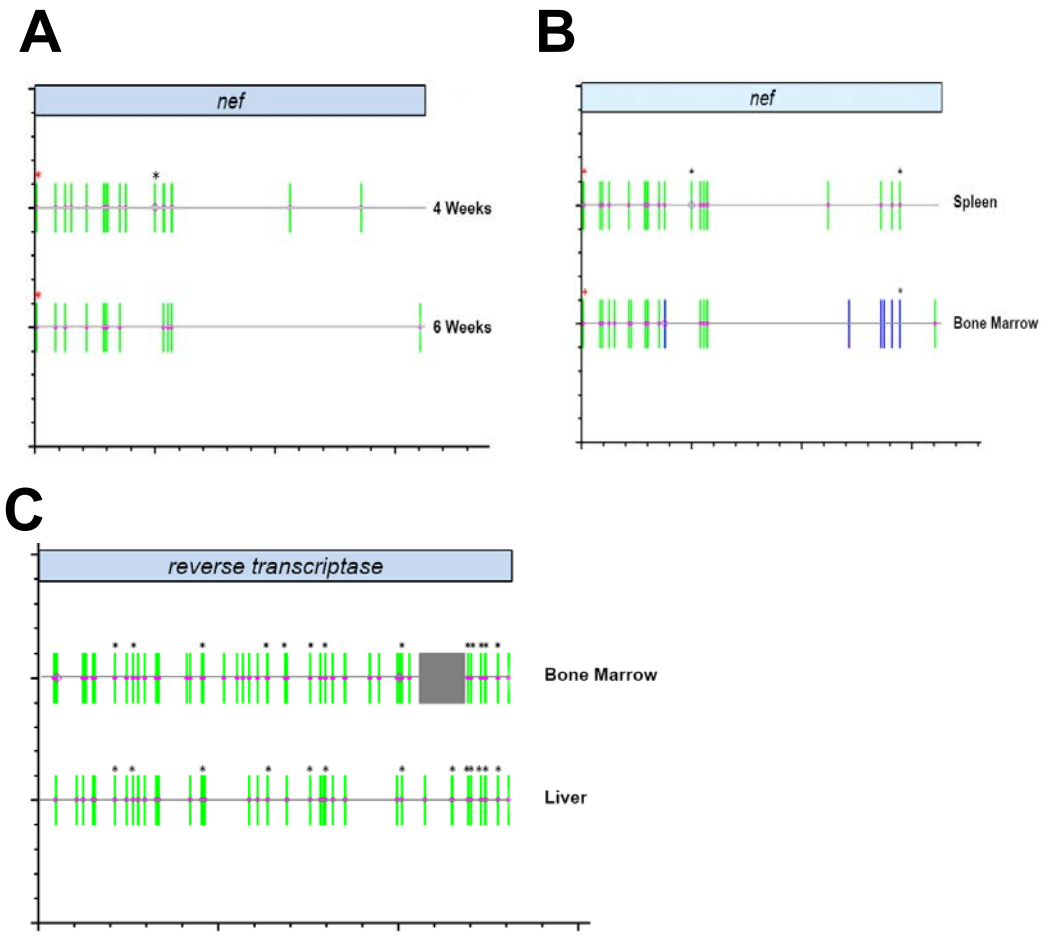


Figure 5.15 Different populations of APOBEC mutated viral DNA detected at multiple time points in the peripheral blood and in multiple tissues.

(A) comparison of Nef sequences revealed two different populations of hypermutated viral DNA detected in the peripheral blood of FS9 at 4 and 6 weeks post infection including mutations disrupting the initiation codon at both time points (red asterisks). (B) Hypermutated Nef DNA present in the spleen and bone marrow. Six G to A changes in the sequence from bone marrow are heterogeneous (blue lines), indicating that multiple populations of viral DNA are present. (C) Reverse transcriptase region of the pol gene amplified from viral DNA in the bone marrow and liver is heavily mutated by APOBEC, with 8 and 13 termination codons introduced respectively (black asterisks). A large deletion is also present in the sequence from the bone marrow (grey box).

Interestingly, the viral sequence from the bone marrow was heterogeneous, with 6 G to A mutations being present only in a portion of the sequence (blue lines), indicating that multiple viral populations are present in this tissue. The reverse transcriptase was also amplified from cells isolated from the bone marrow and liver of this mouse.
The sequences were again extensively mutated by APOBEC with 54 and 48 G to A mutations in the sequences from the bone marrow and liver respectively (Figure 4.15c). These APOBEC mutations created 8 nonsense mutations in the viral DNA from the bone marrow and 13 termination codons in the viral DNA from the liver. In addition, the sequence from the bone marrow had a 124 nucleotide deletion in the 3' half of RT, further showing that the viral DNA recovered from this mouse is very likely replication incompetent.

No replication competent virus recovered from FS6 or FS9

In the two mice that showed evidence of viral replication with viral DNA in peripheral blood and in tissues, I wanted to address whether there was a reservoir of cells harboring replication competent virus. To

determine this, $1x10^6$ cells from the lung of FS6 and from the spleen and bone marrow of FS9, all of which were shown to be positive for viral DNA, were co-cultured with PHA activated human PBMCs. These mice were chosen, as viral DNA was also detected in the peripheral blood, suggesting viral replication had been occurring in these mice. The culture supernatant was sampled at days 5, 9, and 21 and was analyzed for p24 as a marker for viral propagation. Cells from a humanized mouse infected with wild type HIV-1_{JR-CSF} were used as a positive control. By day 5, the culture supernatant for the positive control had over 600 pg/ml of p24 (Figure 4.20). The co-culture supernatants from the cells from HIV-1_{JR-CSF} vif(FS) mice were negative through 21 days of analysis, demonstrating the lack of replication competent virus cells isolated from these tissues. These results demonstrate that the heavily mutated HIV present in the tissues of these mice represents non-replicating, defective proviruses.

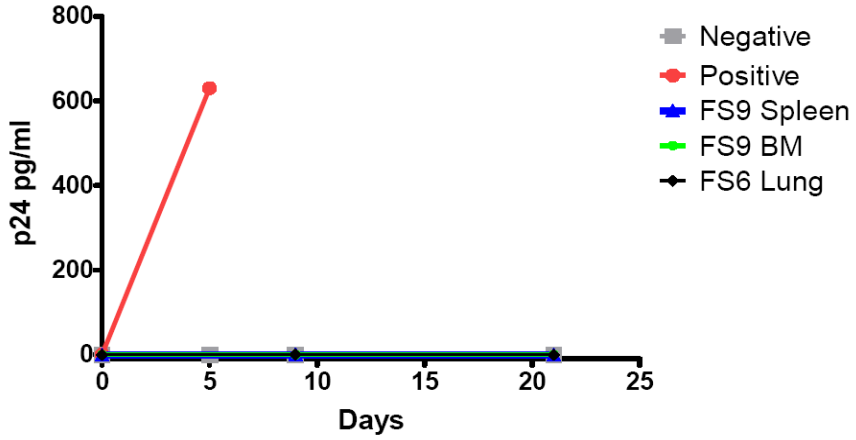


Figure 4.16 No replication competent virus recovered from HIV-1_{JR-CSF} vif(FS) infected mice.

Tissues from two mice, FS5 and FS2, that were found to be positive for viral DNA were co-cultured with PHA activated human PBMC to recover any replication competent virus

Strong in vivo selective pressure on HIV to restore Vif

In the remaining three humanized mice exposed to $3.6x10^5$ TCIU of HIV-1_{JR-CSF} vif(FS), viral breakthrough was observed. In two of the mice, FS11 and FS12, plasma antigenemia was detected demonstrating that viral replication is occurring (Figure 4.17a and 4.19a). Analysis of a 1.4kb region of the viral DNA from nucleotides 4947-6372 which includes the *vif* gene from the peripheral blood of both mice revealed a one base deletion in *vif* (grey line), restoring the open reading frame (Figure 4.17b and 4.19b left panel). Additionally, no APOBEC mutations were present in this 1.4kb section of the provirus from peripheral blood cells at any of the time points analyzed, showing that the virus was not subjected to APOBEC restriction. Interestingly, in mouse FS12, viral DNA is first detected in the peripheral blood 5 weeks post infection revealing a repaired *vif* open reading frame, however viral antigenemia is not observed until 9 weeks post exposure. Viral load analysis of mouse FS12 detected viral RNA in the plasma at 3 weeks post infection (Table 4.2). Likewise, in mouse FS11, Viral antigenemia was not detected in the plasma at 6 and 8 weeks, however, viral RNA is present in the plasma at 6 weeks (Table 4.2). These observations highlight the importance of using multiple assays to detect infection.

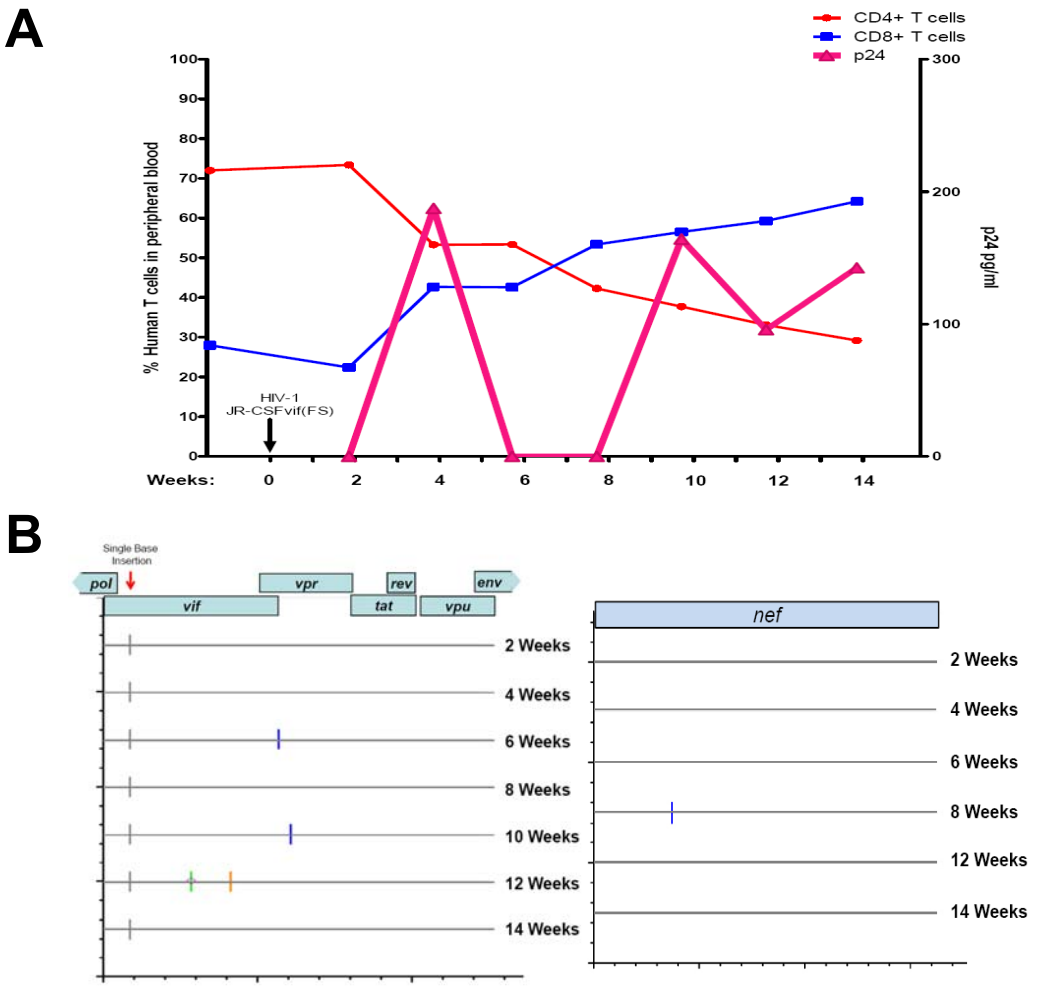


Figure 4.17 Repair of *vif* reading frame in a mouse infected with HIV-$1_{JR\text{-}CSF}$ vif(FS).

(A) Analysis of the peripheral blood of mouse FS11 infected intravenously with Vif frame shifted HIV-1 shows viral antigenemia (pink triangles) coinciding with the loss of $CD4^+$ T cells (red circles). (B) Viral DNA sequence from the peripheral blood at the indicated time points of a 1.4kb region from nucleotides 4947-6372 (left) or of the nef gene at the 3' end of the genome (right). The frameshifted *vif* gene in the 1.4 kb region shows a one nucleotide deletion (grey line) that restores the open reading frame at all time points. In both regions of the genome analyzed, no APOBEC G to A hypermutation is observed at any time point.

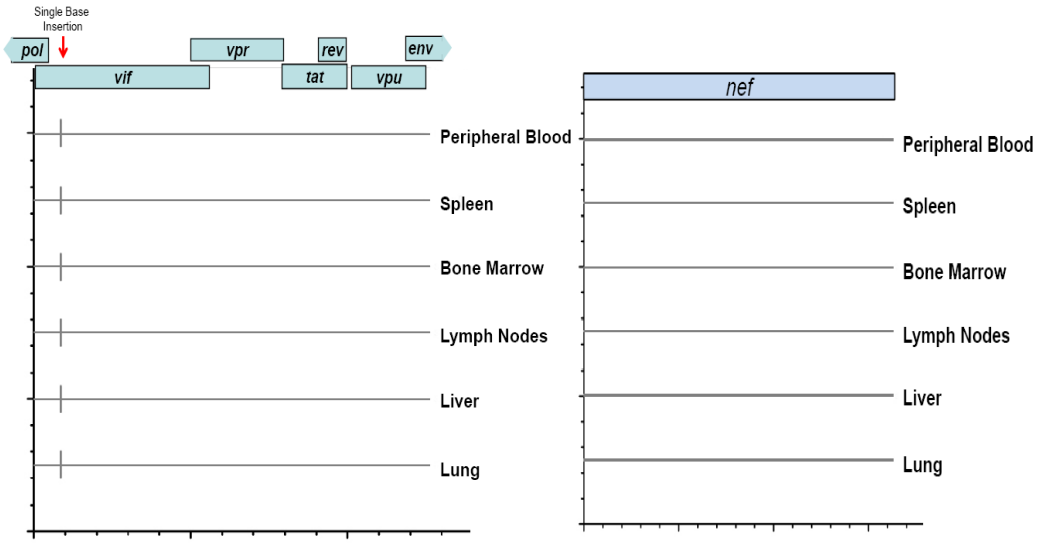


Figure 4.18 Viral DNA from the tissues of Vif restored HIV infected mouse show no APOBEC hypermutation.

The restored *vif* open reading frame (left panel) resulting from a one base deletion (grey line) is present in viral DNA sequences from all tissues in mouse FS11. A 1.4 kb region of the viral DNA from nucleotides 4947-6372 (left) or the nef gene from nucleotides 8721-9441 (right) sequenced show no G to A hypermutation characteristic of APOBEC in any tissue.

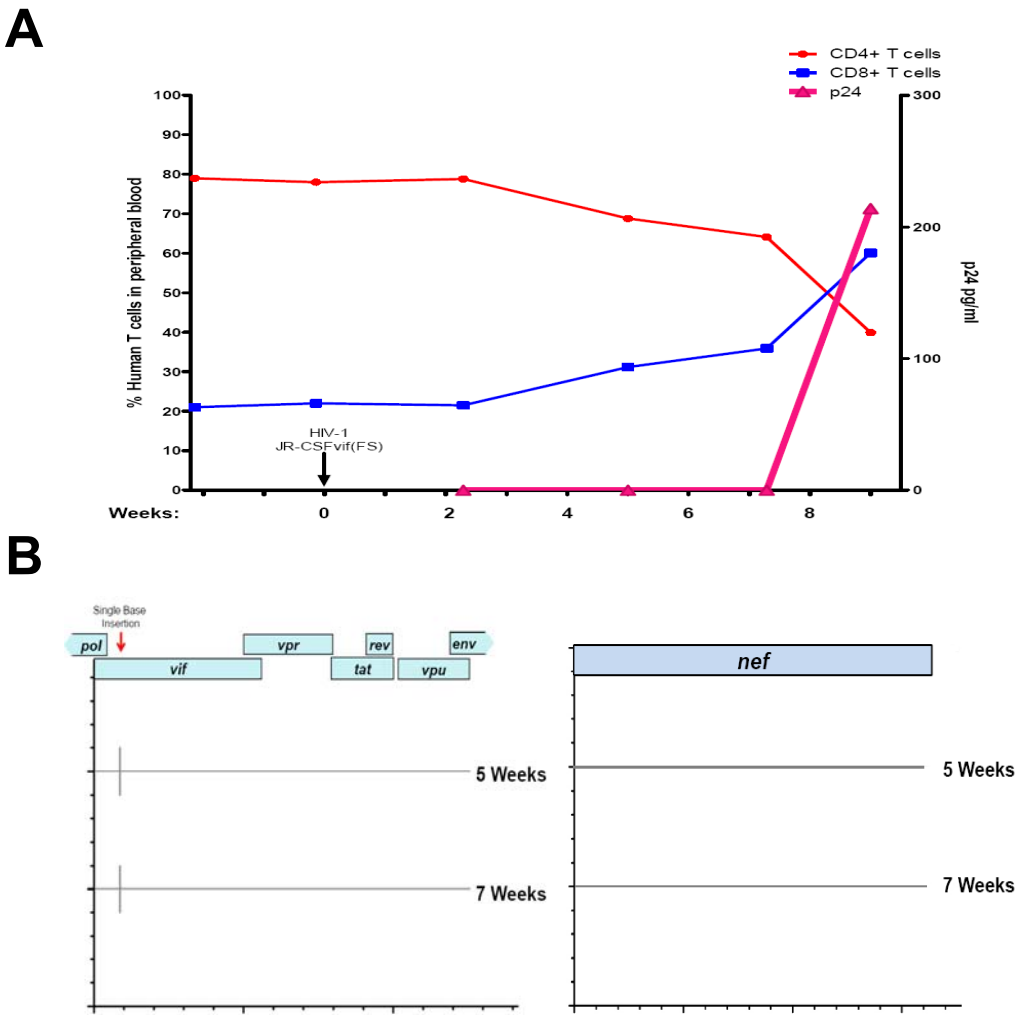


Figure 4.19 Vif repair in a second mouse infected with HIV-1$_{JR\text{-}CSF}$ vif(FS).

(A) Analysis of the peripheral blood of mouse FS12 infected intravenously with Vif frame shifted HIV-1 shows viral breakthrough at 9 weeks post exposure measured by viral antigenemia (pink triangles) coinciding with the loss of CD4$^+$ T cells (red circles). (B) Viral DNA sequence from the peripheral blood at the indicated time points of a 1.4kb region from nucleotides 4947-6372 (left) or of the nef gene at the 3' end of the genome (right). The frameshifted *vif* gene in the 1.4 kb region shows a one nucleotide deletion (grey line) that restores the open reading frame at all time points. In both regions of the genome analyzed, no APOBEC G to A hypermutation is observed at any time point.

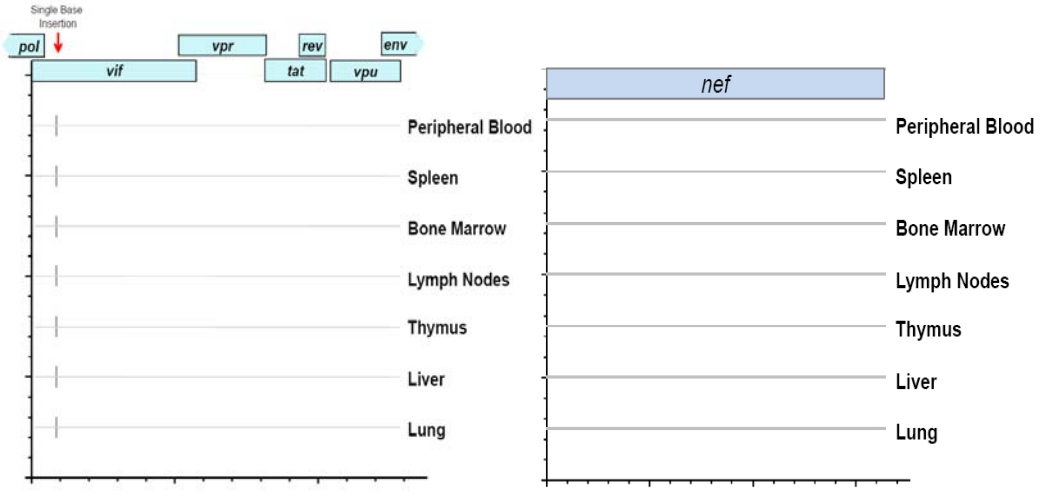


Figure 4.20 Viral DNA from the tissues of a second Vif restored HIV infected mouse show no APOBEC hypermutation.

The restored *vif* open reading frame (left panel) resulting from a one base deletion (grey line) is present in viral DNA sequences from all tissues in mouse FS12. A 1.4 kb region of the viral DNA from nucleotides 4947-6372 (left) or the nef gene from nucleotides 8721-9441 (right) sequenced show no G to A hypermutation characteristic of APOBEC in any tissue.

In one of the mice, FS11, nucleotide changes were detected at single time points, specifically an A to T mutation at 6 weeks, a C to A change at 10 weeks in a population of the viral DNA present highlighted by blue lines in the alignments; also an A to G change along with a single G to A change that is not at an APOBEC restriction site (GCA to ACA) at 12 weeks post infection (Figure 4.17b, left panel). These mutations likely represent errors in reverse transcription and do not remain in the viral population, as they are not observed at any subsequent time points analyzed.

Analysis of the *nef* gene at the 3' end of the provirus from peripheral blood of both FS11 and FS12 likewise revealed no G to A mutations characteristic of APOBEC mutagenesis (Figure 4.17b and 4.19b right panel). In mouse FS11, as with the 1.4 kb region of viral DNA analyzed, a C to A nucleotide change is observed in a population of viral DNA from peripheral blood at 8 weeks post infection likely due to a Reverse Transcriptase error (Figure 4.17b, right panel). Nested PCR for viral DNA in tissues from these mice showed viral DNA present in all tissues analyzed, as seen in humanized mice infected with wild type virus, consistent with active HIV replication. Sequence analysis of the viral DNA from the tissues showed the restored *vif* reading frame and no G to A

mutations in sequences from all tissues, demonstrating the superior fitness of the Vif-repaired virus as no viral DNA containing a disrupted *vif* reading frame was detected (Figure 4.18 and 4.20).

In the third mouse that showed viral breakthrough, FS13, the appearance of plasma antigenemia was delayed for 12 weeks (Figure 4.21). Viral DNA from peripheral blood cells first appeared 6 weeks post infection with numerous G to A mutations present in a 1.4kb region of the viral genome from nucleotides 4947-6372 which includes the *vif* gene, however, sequences from peripheral blood at subsequent time points were found to be heterogeneous as the majority of G to A mutations were only present in a portion of the DNA sequences present (dark blue lines), suggesting that virus replication was occurring (Figure 4.22a). One T to C change present at week 8 that is not observed at subsequent time points, likely represents an error in reverse transcription. Sequence analysis of the *vif* gene from peripheral blood cells confirmed that the insertion disrupting Vif was still present. Viral DNA from peripheral blood at the 3' end of the genome shows a similar pattern of heterogeneous G to A mutations present at weeks 6-10, with only a few APOBEC changes in all of the sequences (Figure 4.22c). At 12 weeks post infection, when plasma antigenemia first appears, analysis of the *vif* DNA sequence

showed a minor population of viral DNA with a one nucleotide deletion that restores the *vif* open reading frame (Figure 4.22b). At subsequent time points, the only viral DNA sequence present has a one base deletion in *vif* (grey line), and two G to A mutations at putative APOBEC sites in the vpu/env region, showing that the virus that was able to repair the *vif* open reading frame was also subjected to a sub lethal level of mutagenesis by APOBEC (Figure 4.22a).

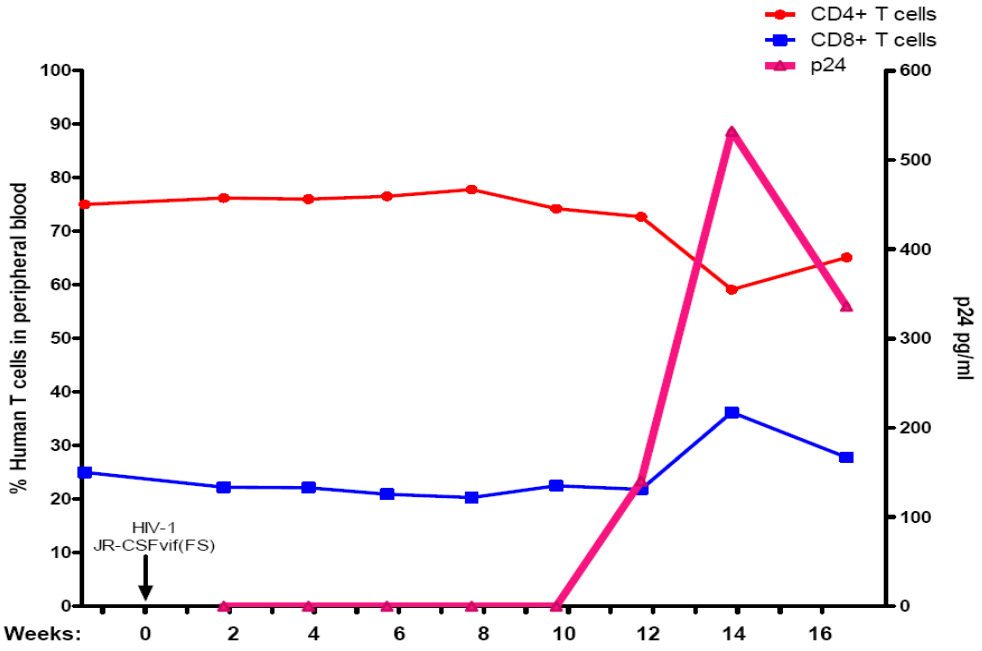


Figure 4.21 Delayed viral breakthrough in a humanized mouse infected with HIV-$1_{JR\text{-}CSF}$ vif(FS).

Analysis of the peripheral blood of mouse FS13 infected intravenously with Vif frame shifted HIV-1 shows viral breakthrough at 12 weeks post exposure measured by viral antigenemia (pink triangles) accompanied by a reduction in $CD4^+$ T cells (red circles).

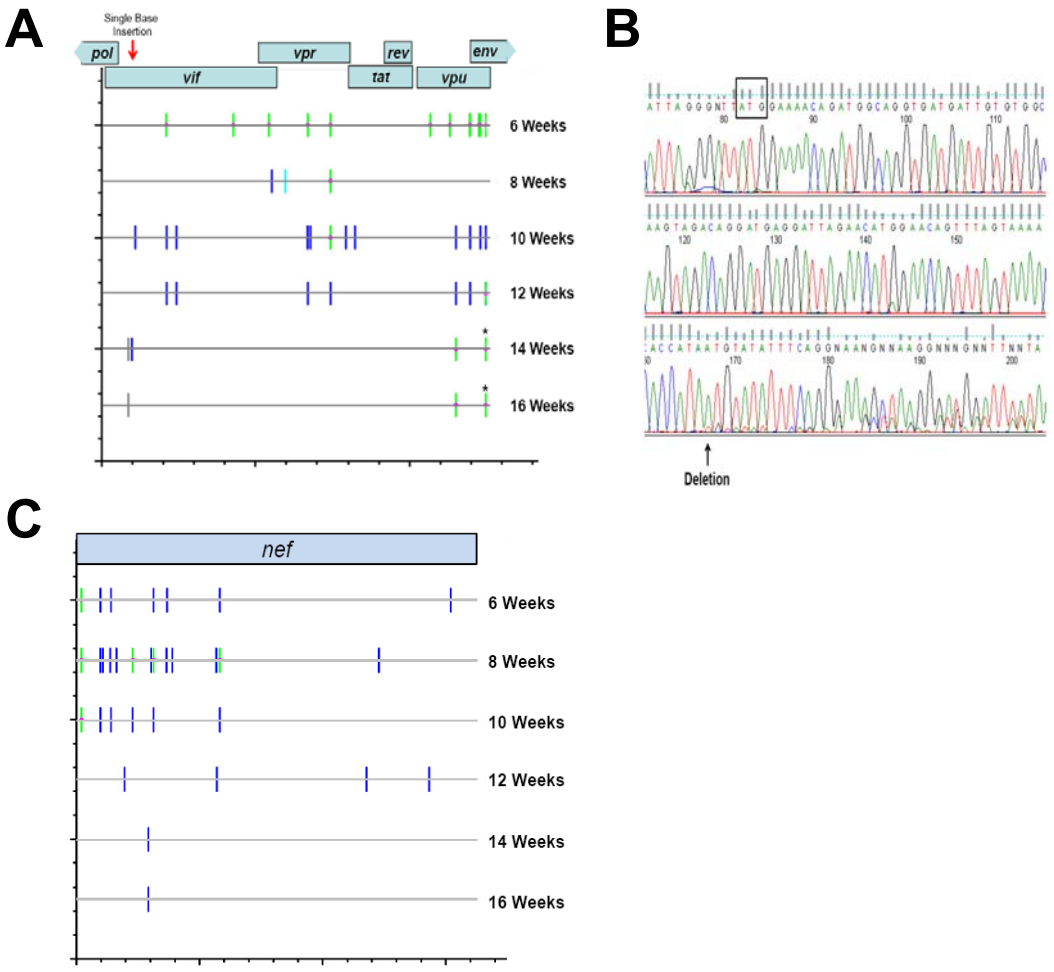


Figure 4.22 Repair of *vif* reading frame at 12 weeks post infection.
(A) Viral DNA alignment from nucleotides 4947-6372 from peripheral blood shows APOBEC hypermutation occurring at early time points, with G to A mutations becoming heterogeneous at 8-12 weeks and a repaired vif reading frame (grey line) appearing at 14 weeks with APOBEC mutations remaining. (B) Viral DNA sequence from 12 weeks post infection of the vif gene (start codon in black box) showing a 1 base deletion present in a minor population. (C) Nef region of viral DNA from peripheral blood shows G to A mutations, most of them heterogeneous (blue lines), at multiple time points.

Interestingly, one of the remaining G to A changes results in a G to E amino acid substitution in the Env protein and introduces a premature stop codon in Vpu, truncating the protein by 7 amino acids (Figure 4.22a asterisks). Analysis of the this 1.4 region of the viral DNA from tissues and also from RNA isolated from cell free virus in the plasma showed that these two APOBEC mutations in the vpu/env genes are present in all sequences (Figure 4.23), showing this virus that repaired Vif was fit for replication and was able to outgrow all other Vif-defective viruses as the sequence from this virus is the only one present. One G to A change not at a putative APOBEC restriction site (GTG to ATG) present in a population of viral DNA from the liver (blue line) likely represents an error in reverse transcription (Figure 4.23 left).

Analysis of Nef at the 3' end of the viral DNA from peripheral blood at weeks 14 and 16 and from the tissues shows no APOBEC mutations present following the appearance of the vif reading frame. The sequences reveal one G to A change not at a putative APOBEC restriction site (GTA to ATA) resulting in a valine to isoleucine amino acid substitution found in all tissues as well as in the RNA from the cell free virus (Figures 4.22c and 4.23 right). Interestingly, in the peripheral blood at 14 and 16 weeks as well as in the lymph nodes and thymus, this change is heterogeneous,

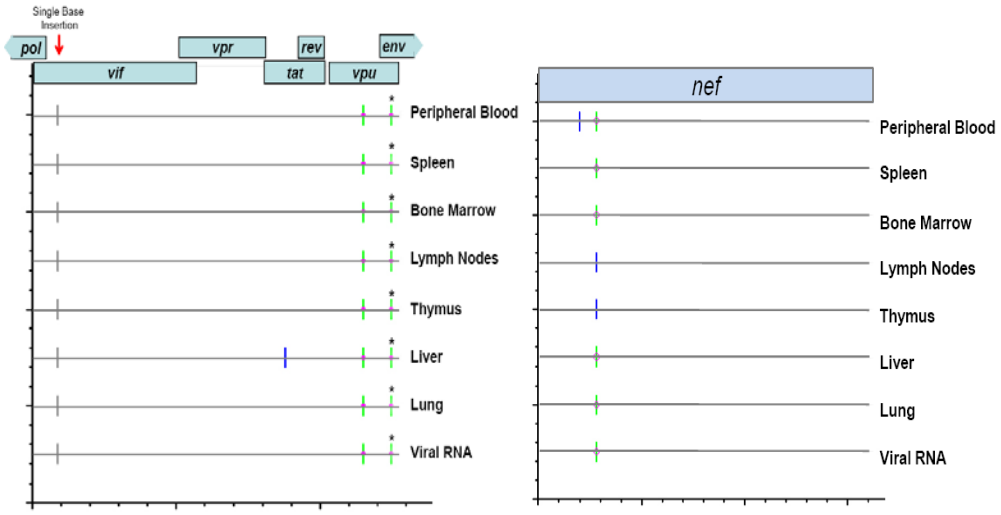


Figure 4.23 Viral DNA from the tissues of a Vif restored HIV infected mouse show few APOBEC mutations.

The restored *vif* open reading frame (left panel) resulting from a one base deletion (grey line) is present in viral DNA sequences from all tissues in mouse FS13. Two G to A changes characteristic of APOBEC mutagenesis are present in all tissues and in the viral RNA from the cell free virus in the plasma in a 1.4 kb region of the viral DNA from nucleotides 4947-6372 (left). The second G to A mutation introduces a stop codon that truncates Vpu by 7 amino acids (black asterisk). Analysis of the 3' region of the viral DNA from nucleotides 8721-9441 (right) sequenced show one G to A change not characteristic of APOBEC in all tissues (Green line with pink diamond, blue line in lymph nodes and thymus).

suggesting an error in reverse transcription that has spread beyond the localized region in which it occurred. In addition, an A to T change is found only in the peripheral blood in a population of viral DNA, again suggestive of an error in reverse transcription (Figure 4.23 right).

The restoration of the *vif* open reading frame in three separate infections demonstrates the strong *in vivo* selective pressure HIV faces to maintain Vif as plasma antigenemia was not detected in any of the mice until after the virus repaired the *vif* reading frame. In all three instances, the Vif expressing virus was no longer subjected to APOBEC mutagenesis, as no G to A mutations appeared in the viral DNA following the appearance of the restored *vif* gene. The reverted virus outgrew all other viral populations and was the only viral sequence detectable in all tissues analyzed demonstrating the fitness of Vif-expressing HIV in vivo and further highlighting the deleterious effects of APOBEC on the virus in the absence of Vif.

Mice exposed to four-fold less HIV-1_{JR-CSF} vif(FS)

After demonstrating the strong selective pressure on HIV to maintain Vif, I wanted to determine whether the ability of HIV-1_{JR-CSF} vif(FS) to restore the vif open reading frame was dependent on the initial infectious dose. To address this, five humanized mice were injected intravenously with $9x10^4$ TCIU of HIV-1_{JR-CSF} vif(FS), a four-fold reduction in the infectious units, and the mice monitored for evidence of viral replication in the periphery. In 4 of the mice no viral antigenemia was detected at any of the time points analyzed and no loss of $CD4^+$ T cells was observed in the peripheral blood (Figure 4.24).

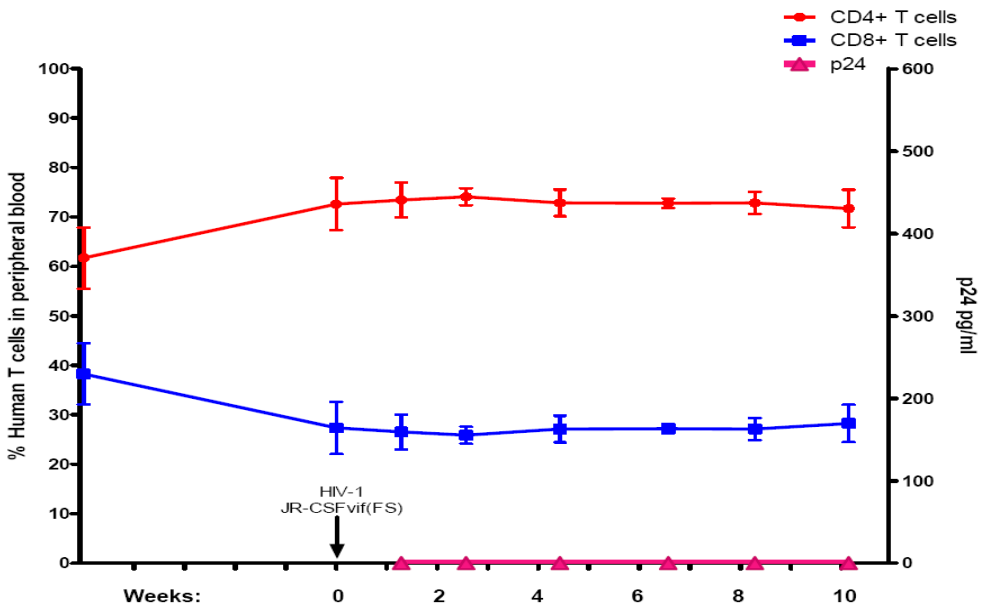


Figure 4.24 Mice exposed to a four-fold reduced infectious dose HIV-1_{JR}.CSFVif(FS) show no evidence of viral replication or $CD4^+$ T cells loss in the peripheral blood.

Monitoring of peripheral blood of 4 humanized mice exposed intravenously to $9x10^4$ TCIU of HIV-$_{1JR-CSF}$vif(FS) showed no loss of $CD4^+$ T cells and no viral antigenemia at any time point analyzed.

In addition, no viral DNA was found using two separate nested PCR analyses of peripheral blood cells showing a lack of viral DNA in the periphery. Analysis of the tissues from these mice for viral DNA using nested PCR primers for *nef* and *reverse transcriptase* revealed that two mice, FS15 and FS17, harbored proviral DNA in cells isolated from lungs. No viral DNA was found in any other tissue from these mice. Analysis of the tissues from the other two mice showed no detectable viral DNA by two separate nested PCR reactions.

Sequence analysis of the amplified viral DNA showed extensive APOBEC mutagenesis in all sequences. The reverse transcriptase region of the *pol* gene from viral DNA recovered from the cells isolated from the lungs of both mice showed extensive G to A hypermutation with 36 changes in viral DNA from FS15 (10 of them resulting in premature termination codons from tryptophans). The sequence from mouse FS17 had 41 G to A mutations (12 of them as nonsense mutations disrupting Reverse Transcriptase) (Figure 4.25a). Viral DNA from the 3' region of the genome encompassing the *nef* gene from mouse FS17 was also found to have 19 G to A mutations, with two of them disrupting Nef, one mutation changing the initiation codon to isoleucine (red asterisk) and a

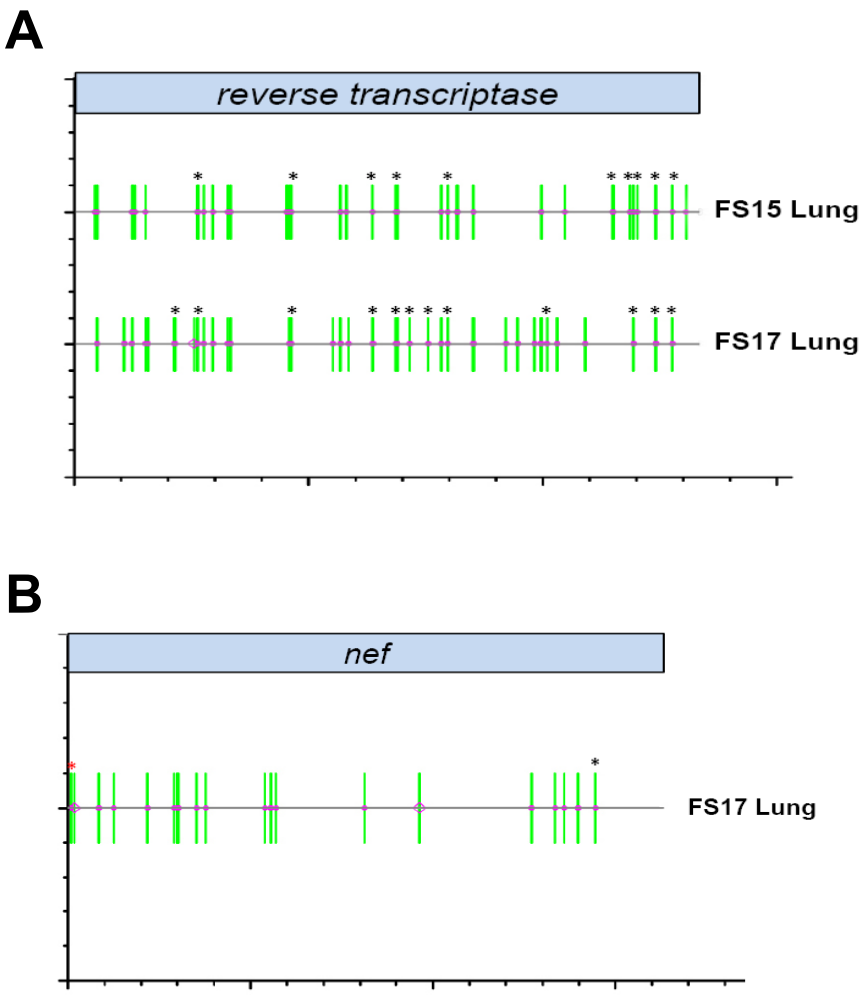


Figure 4.25 APOBEC hypermutated viral DNA recovered from the lung of two mice infected with HIV-$1_{JR\text{-}CSF}$Vif(FS).

(A) Analysis of the RT sequence from viral DNA recovered from the lung of FS15 and FS17 shows extensive APOBEC hypermutation, with 10 and 12 termination codons (black asterisks) introduced respectively. (B) The Nef region of the viral DNA isolated from the lung of mouse FS17 showing extensive APOBEC hypermutation with both the initiation codon disrupted (red asterisk) and one premature stop codon (black asterisk).

second mutating a tryptophan codon to a stop codon (black asterisk) (Figure 4.25b). The *nef* gene from the viral DNA in the cells from the lung of mouse FS15 could not be amplified in three separate reactions.

The fifth humanized mouse receiving an intravenous injection of $9x10^4$ TCIU of HIV-1_{JR-CSF} vif(FS) showed viral breakthrough in the form of viral antigenemia by 10 days post exposure, the first time point analyzed (Figure 4.26a). Plasma p24 persisted with a continuous loss of $CD4^+$ T cells throughout the course of the infection. Viral RNA was also found to be present in the plasma of FS14 at all time points analyzed (Table 4.2). Viral DNA was present in cells isolated from the peripheral blood beginning at 10 days post infection. Sequence analysis of a 1.4kb region of the viral genome from nucleotides 4947-6372 which includes the *vif* gene showed a one nucleotide deletion at the location of the insertion (grey line), restoring the *vif* open reading frame to the wild type sequence (Figure 4.26b left). No G to A changes in the viral DNA from peripheral blood in this 1.4 kb region or from the 3' region including the *nef* gene (Figure 4.26b right) were observed at any time point, consistent with Vif expression. The appearance of the restored *vif* reading frame along with absence of G to A mutations in the viral DNA sequences suggests that the

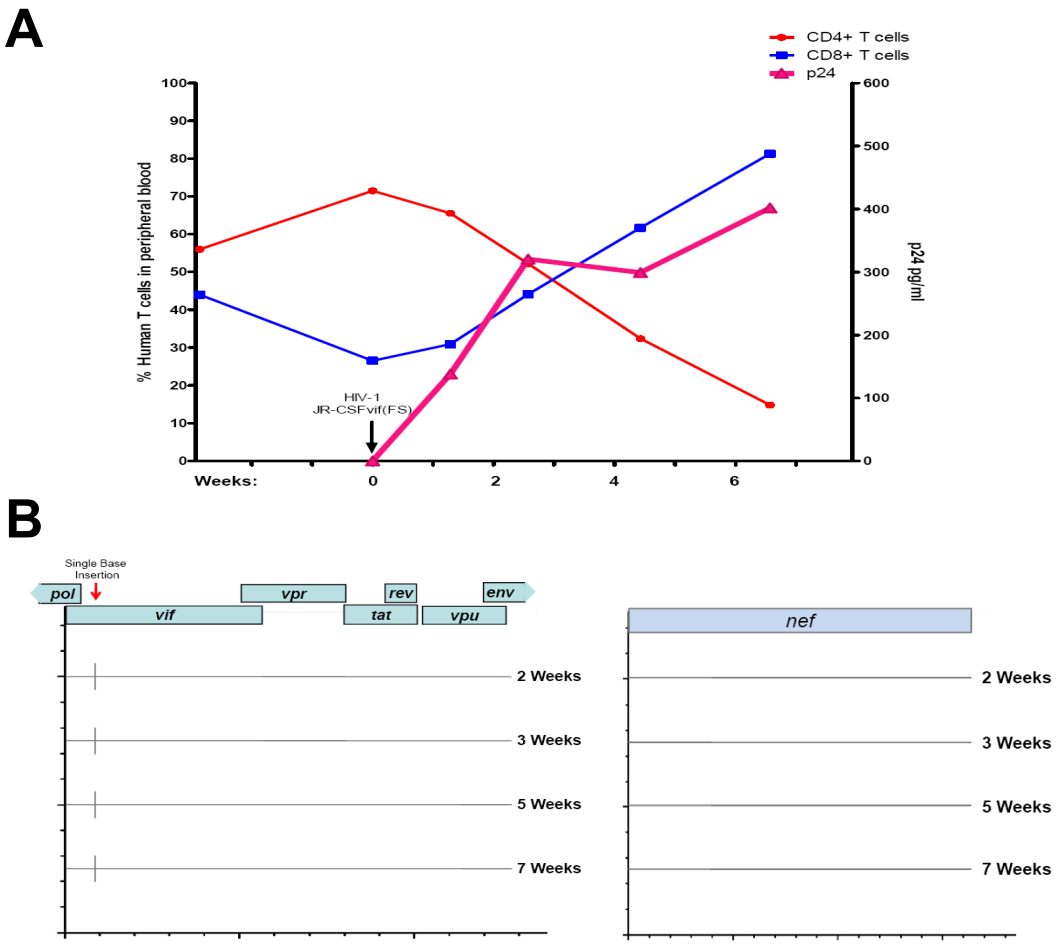


Figure 4.26 Vif repair in a mouse infected with $9x10^4$ TCIU of HIV-1_{JR-CSF} vif(FS).

(A) Analysis of the peripheral blood of mouse FS14 infected intravenously with Vif frame shifted HIV-1 shows viral breakthrough within 2 weeks post exposure measured by viral antigenemia (pink triangles). Antigenemia continues to increase as infection progresses accompanied by a loss of $CD4^+$ T cells (red circles). (B) Viral DNA sequence from the peripheral blood at the indicated time points of a 1.4kb region from nucleotides 4947-6372 (left) or of the nef gene at the 3' end of the genome (right). The frameshifted *vif* gene in the 1.4 kb region shows a one nucleotide deletion (grey line) that restores the open reading frame at all time points. In both regions of the genome analyzed, no APOBEC G to A hypermutation is observed at any time point.

repair of Vif occurred in the first few rounds of replication, preventing APOBEC restriction .

Tissues were collected from this humanized mouse eight weeks post infection and analyzed for the presence of viral DNA by nested PCR for both the 1.4 kb region from nucleotides 4947-6372 and the Nef region at the 3' end of the genome. Viral DNA was present in all tissues analyzed with the exception of the spleen, which was found to be grossly necrotic at the time of harvest. Consistent with results from previous mice in which the virus restored Vif, proviral DNA sequences in the 1.4 kb from nucleotides 4947-6372 showed the one base deletion in *vif* that restored the open reading frame is present in all tissues with no G to A mutations observed (Figure 4.27 left). The sequences of the 3' region including *nef* also had no G to A mutations in any tissue (Figure 2.27 right) demonstrating that the restoration of the *vif* open reading frame protected the virus from APOBEC restriction.

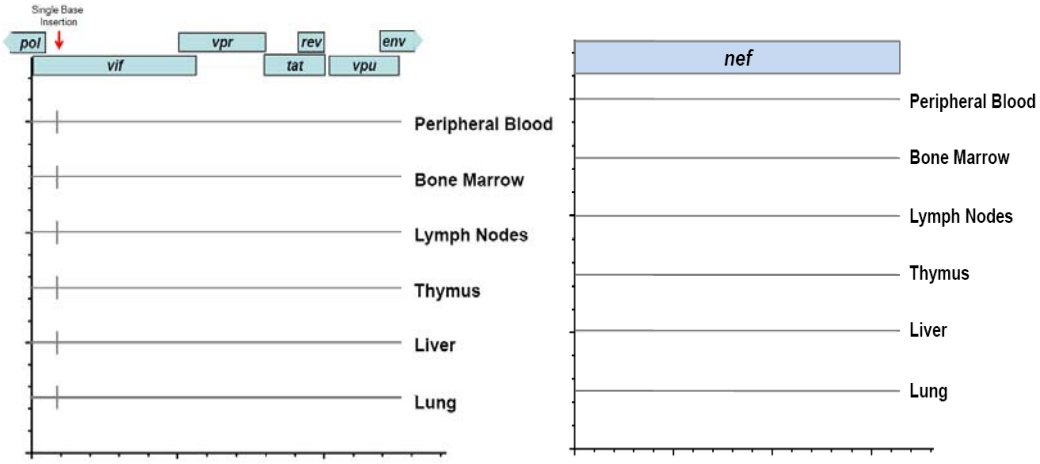


Figure 4.27 Vif restored viral DNA from a mouse infected with $9x10^4$ TCIU of HIV-1_{JR-CSF} vif(FS) was present in all tissues and not subjected to APOBEC hypermutation.

The restored *vif* open reading frame (left panel) resulting from a one base deletion (grey line) is the only viral DNA sequence present in tissues from mouse FS14. A 1.4 kb region of the viral DNA from nucleotides 4947-6372 (left) or the nef gene from nucleotides 8721-9441 (right) sequenced show no G to A hypermutation characteristic of APOBEC in any tissue.

Summary

HIV-1_{JR-CSF} that is deficient in Vif is susceptible to restriction by APOBEC, however, in cell lines that lack APOBEC proteins, the Vif-defective viruses are able to replicate similar to wild type HIV and no G to A hypermutation occurs. In humanized mice, wild type HIV-1_{JR-CSF} injected intravenously is found in all tissues analyzed as early as one week post infection. Sequence analysis of the viral DNA showed no G to A hypermutation. HIV-1_{JR-CSF} that is defective in Vif, either by a non-revertible deletion or a one base insertion, is severely crippled in its ability to replicate *in vivo* as viral DNA is found only in limited tissues one week post infection. Furthermore, HIV-1_{JR-CSF} with a deletion in vif that cannot be reverted is lethally restricted by APOBEC, as no evidence of viral replication is seen in the periphery of the humanized mice at any time point after infection by analysis for both viral RNA and antigenemia. Analysis of viral DNA found in the tissues of these mice only shows hypermutated proviruses in a few tissues. In some cases no viral DNA was found in any of the tissues analyzed. Likewise, APOBEC lethally restricted the virus with a frameshift in *vif* with only heavily mutated viral DNA recovered from the humanized mice. In some cases HIV was able to restore the *vif* open reading frame with a one base deletion. Following the

restoration of Vif, the course of infection resembled that of wild type HIV, with viral antigenemia detected and viral DNA present in all tissues analyzed indicating an actively replicating virus. Importantly, following the restoration of Vif, no new G to A mutations are found in the viral DNA, demonstrating that the Vif-repaired virus is no longer susceptible to restriction by APOBEC.

CHAPTER FIVE: Conclusions and Recommendations

For my studies I focused on an issue of fundamental importance to the field of HIV, namely what is the *in vivo* role of Vif, a protein encoded by one of the auxiliary genes of HIV. Since Vif's main activity is to antagonize cellular restriction factors APOBEC3G and 3F , I characterized the expression of these proteins in humanized mice to determine if they could serve as an *in vivo* model to investigate the Vif/APOBEC axis. Quantitative real time PCR analysis (Vetter, Johnson et al. 2009) demonstrated that human APOBEC3G and 3F are expressed in the spleen, bone marrow, lymph node, liver and lung, thus confirming that human APOBEC3G and 3F are expressed in the primary, secondary and immune effector organs of humanized mice. To investigate the effect of human APOBEC on HIV replication in vivo I used a primary strain of virus designated JR-CSF originally cloned from the CSF of an infected patient (Koyanagi, Miles et al. 1987). HIV-$1_{JR\text{-}CSF}$ is a CCR5-tropic replication competent virus with a complete complement of fully functional open reading frames for all HIV encoded genes.

Vif-deficient HIV-1_{JR-CSF} replicates in permissive cells

First I fully characterized the expression and functionality of the Vif gene of this virus *in vitro*. Vif is expressed after transfection of the provirus into 293T cells and is fully capable of inducing the degradation of exogenous APOBEC, however transfected provirus from Vif disrupted (frame shift or deletion) HIV-1 did not result in Vif protein expression and no degradation of exogenous APOBEC3G was observed. Secondly, I compared the replication of Vif-deficient HIV-1_{JR-CSF} to the replication of wild type HIV using a permissive (APOBEC deficient) cell line. My results demonstrated that disruption of the Vif ORF (frame shift or deletion) does not affect the propagation of HIV in vitro, as these Vif-defective viruses were capable of replicating like wild type viruses in permissive cells. Together, these results confirmed the potential of HIV-1_{JR-CSF} to investigate the role of Vif in virus replication and pathogenesis in an *in vivo* system.

HIV-1$_{JR-CSF}$ infection of humanized mice leads to high levels of virus replication and depletion of human CD4+ T cells.

Active viral replication in humanized mice intravenously infected with wild type HIV-1$_{JR-CSF}$ showed high levels of viral RNA and antigenemia in the plasma. In addition, peripheral blood cells from all infected animals contained viral DNA as determined by quantitative real time PCR analysis. A consistent and reproducible reduction in the levels of CD4+ human T cells was observed in the peripheral blood of all animals infected with HIV-1$_{JR-CSF}$. A more comprehensive analysis of the lymphoid and non-lymphoid tissues of HIV infected humanized mice demonstrated the presence of actively replicating viruses in all tissues analyzed. Sequence analysis of viral DNA recovered from either peripheral blood or tissues obtained from humanized mice infected with HIV-1$_{JR-CSF}$ demonstrated little sequence variation over weeks or months and for the most part no G to A changes attributable to APOBEC enzymes were noted.

Vif-deleted HIV-1_{JR-CSF} is lethally restricted by APOBEC *in vivo*

Once I had established the replication of wild type HIV-1_{JR-CSF} and the background of sequence mutations in humanized mice I proceeded to evaluate the replication capacity of a virus containing a deletion in Vif. This deleted virus is incapable of expressing Vif *in vitro* and because of the extent of this particular deletion it would not be expected to revert to contain a functional protein. When humanized mice were exposed intravenously to a high dose of Vif deleted HIV-1_{JRCSF}, the virus was found to be severely attenuated. This was reflected in the very low and sporadic levels of viral DNA found in a few tissues one week after inoculation that contrast with the high levels and broad distribution of viral DNA observed in animals infected with wild type virus. In contrast to the viral sequences obtained from animals infected with wild type virus, analysis of the viral DNA sequences recovered from mice infected with the virus containing a deletion in Vif showed high levels of characteristic G to A mutations demonstrating strong APOBEC restriction in vivo. The severity of these extensive mutations was reflected in the lack of evidence of virus replication as determined by an absence of viral RNA in plasma and the absence of viral DNA in peripheral blood cells at any of the multiple time points analyzed in all of the exposed mice. However, when tissues from

exposed animals were analyzed for the presence of viral DNA, I found it to be present only in the lung in 3/5 mice and in the lymph nodes of one of these three mice. No viral DNA was found in any of the tissues analyzed from 2/5 exposed mice. In the few cases where viral DNA was found, sequence analysis showed extensive G to A hypermutation, demonstrating the lethal restriction of HIV by APOBEC in the absence of Vif. It should be noted that unlike what has been reported from in vitro culture experiments (Hache, Shindo et al. 2008) we did not detect any compensatory mutations that could overcome the loss of Vif and give rise to a replication competent virus. These results represent strong evidence in support of Vif as a good candidate for the development of novel inhibitors of HIV.

Strong in vivo selective pressure to restore a functional Vif phenotype

Having established the absolute requirement of Vif for HIV-1 replication *in vivo* and the complete absence of any type of compensatory mutations that could result in a replication competent pathogenic virus, I proceeded to determine the potential for *in vivo* selective pressure to restore a functional Vif. For this purpose I used a virus with a minimal

disruption of the *vif* gene. Specifically I tested the ability to replicate in humanized mice of a virus containing a single point insertion in Vif resulting in a frame shift in the ORF. One week post exposure with HIV-$1_{JR\text{-}CSF}$vif(FS) I did not observe any evidence of viral DNA or RNA in peripheral blood cells or plasma. However I was able to detect viral DNA in a few tissues demonstrating infection. However, the viral DNA present in tissue cells one week post infection contained APOBEC induced mutations.

In six humanized mice that were infected for longer periods of time with this frame shift virus there were no signs of virus replication as determined by two different highly sensitive techniques. However, in two of these six mice I found the transient presence of extensively mutated viral DNA in cells from the peripheral blood two and four weeks after inoculation. Systemic analysis of these mice demonstrated that viral DNA was absent from most tissues. In the cases where viral DNA was found, the proviruses present contained multiple APOBEC induced mutations. In addition attempts made to recover virus from these samples demonstrated that no replication competent HIV was present. These data demonstrate that loss of Vif allows APOBEC to lethally restrict HIV-1 *in vivo* and in

cases where no viral DNA can be found in any tissue, the potential to clear the initial infection.

In four instances, however, mice infected with the frame shift virus demonstrated obvious signs of virus replication in peripheral blood that resembled in many aspects the replication of wild type virus. Specifically, I noted the presence of viral antigenemia and viral RNA in plasma and viral DNA in blood cells. In addition, there was a drop in the levels of CD4+ human T cells in peripheral blood. Sequence analysis demonstrated that the virus actively replicating in these animals had repaired the Vif ORF and none or only a few APOBEC mutations were noted in these proviruses. Thus, the virus was able to restore the vif open reading frame, resulting in an infection similar to wild type HIV, with high levels of plasma antigenemia and viral DNA in all tissues analyzed. In three of these infections the restoration of Vif occurred early, possibly during the first few rounds of virus replication, as no G to A mutations in the viral DNA from these mice were found suggesting that they restored the *vif* open reading frame prior to being subjected to APOBEC mutagenesis. In the fourth infection where the Vif ORF was restored, the repaired vif open reading frame was not seen in the viral DNA until 12 weeks post exposure which was concomitant with the appearance of

plasma antigenemia. APOBEC mutated viral DNA was found in peripheral blood prior to the repair of the frameshift in *vif*, however the sequences were heterogeneous showing multiple highly defective forms of the virus. Following the restoration of Vif, two G to A mutations remained in the viral DNA from every tissue as well as in cell free virus in the plasma, demonstrating that one virus that had been subjected to limited APOBEC mutagenesis but was able to restore Vif prior to being lethally restricted. The observation that the reverted virus did not appear until 3 months after exposure suggests that either a cell harboring a functional provirus had remained in the mouse and was producing virus for an extended period, allowing the virus to ultimately repair the *vif* reading frame or a small amount of replicating Vif deficient virus was able to avoid lethal restriction by APOBEC until it could restore the *vif* open reading frame. These results demonstrate the strong selective pressure on HIV to maintain a functional Vif, as well as the ability of viral reservoirs to allow HIV to persist minimally affected by APOBEC and in limited cases, to escape host restriction.

Together my results provide the first demonstration that human APOBEC can lethally restrict Vif-deficient HIV-1 *in vivo*. As was previously reported from cell culture based systems, all *nef* sequences

analyzed from humanized mice showed no G to A mutations in the 3'ppt despite ample GG and GA target sites (Yu, Konig et al. 2004; Suspene, Rusniok et al. 2006). This observation correlates with the 3'ppt region on the minus strand of the viral DNA being single stranded for a very short period of time, if at all, and thus not being susceptible to APOBEC mutagenesis (Yu, Konig et al. 2004). It is only when HIV-1 can restore the *vif* ORF that significant levels of viral replication and propagation can occur, as no escape mutants developed using second site mutations in the genome to allow viral replication and evasion of APOBEC restriction in the absence of Vif. In addition my results provide *in vivo* validation for HIV-1 Vif as a potentially useful therapeutic target. Nevertheless, additional questions remain to be addressed in future studies.

Evaluation of mutations in Vif that disrupt specific functions of the protein to determine their in vivo relevance

One of the questions that remains to be addressed is the relative contribution of APOBEC3G and 3F to the *in vivo* restriction of HIV. Mutations that have been shown to specifically disrupt Vif binding to either APOBEC3G or APOBEC3F *in vitro* could be used to determine if only one of the APOBEC proteins is sufficient to restrict HIV *in vivo.* Data

generated using *in vitro* systems suggests that APOBEC3G or APOBEC3F can individually restrict Vif-deficient HIV (Sheehy, Gaddis et al. 2002; Zheng, Irwin et al. 2004), however, these experiments rely on ectopic expression of APOBEC in permissive cells. *In vivo*, all APOBEC proteins would be present in the infected cell but the Vif mutant would be partially functional and able to bind either APOBEC3G or APOBEC3F. One could imagine that this could be a scenario encountered with a drug specifically targeting one part or another of the protein. If this drug were to only disrupt binding to either APOBEC 3G or 3F it might not be effective *in vivo*. Alternatively, if a drug or novel compound were capable of interfering with the binding of both APOBEC proteins the remaining question would be whether reversion/mutation of one of the two sites would be sufficient for Vif to regain the ability to prevent restriction of HIV.

The development of small molecule inhibitors of the Vif/APOBEC axis represents novel potential approaches to control HIV infection. However, the rational design of these antivirals must be based on careful confirmation of the *in vivo* relevance of the specific site in the protein being targeted. For example, *in vitro* studies have shown that in order to induce APOBEC degradation, Vif must not only be able to bind the APOBEC proteins, but must also interact with Cullen 5 and elongin C to recruit the

E3 ubitiquin ligase complex. *In vitro* analysis of relevant mutants has determined the putative location of the areas of potential contact and interaction between Vif and its putative partners. Whether these or other putative protein-protein interactions are relevant or significant *in vivo* remains to be determined. My results show that humanized mice can be an outstanding system to begin to address these highly relevant questions. For example, will disruption of Vif recruitment of the E3 components be sufficient to block its ability to protect nascent virions from APOBEC? If so, this approach is an appealing therapeutic target since this would simultaneously prevent Vif from degrading both APOBEC3G and 3F, permitting both proteins to restrict HIV. However, is a mutation introduced at this site likely to revert or to induce compensatory mutations that would restore full function? This is a very realistic possibility given the strong ability of HIV to develop resistance to virtually all drugs and inhibitors currently available (Johnson, Brun-Vezinet et al. 2008).

Although in a very limited way, one study using 3 non-human primates used a SIV-HIV chimera (SHIV) containing a two amino acid mutant aimed at disrupting the interaction between Vif and elongin C to prevent recruitment of the ubitiquin ligase complex (Schmitt, Hill et al. 2008). In this study, the mutant SHIV established infection with 100 fold

lower levels of viremia and with no significant $CD4^+$ T cell depletion when compared to infection with the parental highly pathogenic virus. Interestingly, in 2 of 3 macaques infected with this mutant SHIV, the serine residue that had been originally changed to alanine was mutated by to threonine. The significance of this mutation in Vif remains unclear, however in the two monkeys where this A to T change occurred, a stronger antibody response was noted compared to the monkey where the SHIV did make this change. This mutation made by the virus may represent an initial step in reverting Vif to restore its ability to recruit the ubitiquin ligase complex. Importantly, most G to A mutations observed in the viral DNA recovered from the infected monkeys were in the context of 5'-GA to 5'-AA, characteristic of APOBEC3F. This observation suggests that mutations or drugs targeting Vif recruitment of the ubitiquin ligase complex may not be sufficient to allow APOBEC3G restriction, but will prevent Vif from protecting the virus from APOBEC3F. Additionally, restriction *in vivo* by APOBEC3F may be sufficient to reduce viral replication and quell the cytopathic effects on $CD4^+$ T cells. Since differences between SIV and HIV Vif as well as simian and human APOBEC proteins may reveal a different phenotype additional studies of this type will be necessary to confirm if the APOBEC3F specific restriction also occurs in HIV Vif.

Is there a "sanctuary" where HIV can be protected from APOBEC activities?

One of the most puzzling results obtained was the observation that virus replication in peripheral blood could be observed starting many weeks after the original inoculation. Thus in this mouse there was an occult infection that was likely seeded immediately after the original inoculation but that was not manifested in peripheral blood until 12 weeks later. It should be noted that sequence analysis of the virus present in this mouse only had few mutations induced by APOBEC suggesting that during this prolonged period of quiescence or latency HIV was spared from editing. These results can potentially be explained in several ways. For example, a mutant HIV that had reverted during the first few rounds of virus replication would only have a limited number of (if any) APOBEC induced mutations. This virus could have latently infected a cell that was either quiescent or unable to produce virus. Subsequent activation of this cell could result in the virus spread noted in peripheral blood. The identity and location of the cells harboring viral DNA up to 16 weeks post exposure needs to be determined as it may be related to natural *in vivo*

reservoirs of HIV infection. Determining the location and identity of the cell types that are capable of maintaining reservoirs of virus and their locations will be important, as Vif inhibiting compounds will need to have access to these reservoirs.

Vif inhibitors for antiretroviral therapy

Using humanized mice I demonstrated the lethal restriction of a Vif-defective HIV by APOBEC resulting in abortive infections. However, one issue that remains to be addressed is what will be the effect of inhibiting Vif after infection and after viral reservoirs have been established. This is the scenario that will be encountered by anti-Vif inhibitors in infected patients. One question of high significance is whether pharmacological inhibitors of Vif can halt viral propagation by inducing lethal mutations and in this manner prevent the establishment of new viral reservoirs capable of producing replication functional virus? Vif inhibitors are not expected to prevent virus production from infected cells. They are expected to allow APOBEC encapsidation into nascent virions capable of hypermutating the viral DNA in subsequent infections, eventually resulting in non-functional virus. Preventing the establishment of new viral reservoirs will be essential to eradicating HIV from infected patients, as latently infected

resting $CD4^+$ T cells have a half life up to 44 months (Finzi, Blankson et al. 1999). Targeting Vif early in infection, as a form of post-exposure prophylaxis (PEP) could potentially impact the seeding of these reservoirs making eradication of the virus feasible. Additionally, the potential to target Vif in pre-exposure prophylaxis (PrEP) exists. Specifically, by inhibiting Vif activity prior to the establishment of infection it would be possible to introduce enough mutations that would make these viruses less fit to replicate in the host? Inhibition of Vif at the onset of HIV infection would result in one round of infection, however all nascent virions would have encapsidated APOBEC, thus reducing the number of infections that seed long-lived viral reservoirs. The reduction in the viral reservoir will ultimately allow for the possibility of virus eradication, a concept that seems improbable with current HAART regimens (Shen and Siliciano 2008).

The potential of using Vif as a target for drug development has been established. The first small molecular inhibitor of Vif was recently described as functional in cell culture systems (Nathans, Cao et al. 2008). The authors screened a diverse library of small molecules using 293T cells transfected with YFP tagged APOBEC3G and an HIV provirus (using a Vif-defective provirus as a control). The dual transfected cells were then

treated with the compounds and YFP fluorescence was measured to determine APOBEC3G degradation. One compound, RN-18, preserved APOBEC3G in the presence of Vif, both in their YFP system and by immunoblotting for APOBEC3G. Importantly, since Vif mediated degradation of APOBEC3G occurs through the proteosome, the authors used expression of the cell cycle inhibitor p21 to confirm that RN-18 was not a general proteosome inhibitor. RN-18 was found to increase expression of APOBEC3G in a Vif dependant manner, while simultaneously reducing the half-life of Vif in the presence of APOBEC3G *in vitro*. To date no evidence of RN-18 *in vivo* efficacy has been published. In addition, it is not clear what its mechanism of action is or if it acts directly on Vif or APOBEC. Further improvements on this molecule and development of better inhibitors are very likely. However, more careful and extensive *in vitro* analysis will be needed in order to justify their evaluation *in vivo*.

Determining the most effective method to administer an antiretroviral regimen targeting Vif is an additional consideration that must be addressed. As indicated above, the ability of HIV to develop resistance to drugs is well documented and drug resistance mutations in HIV have been reported for every antiretroviral drug that is presently approved for

treatment (Johnson, Brun-Vezinet et al. 2008). Current therapy for HIV infection consists of three antiretroviral drugs given in combination, referred to as highly active antiretroviral therapy (HAART). Currently available antiretroviral drugs inhibit viral replication that can reduce viremia to undetectable levels in patients. HAART effectively suppresses HIV, however it does not eradicate the virus and discontinuation of antiretrovirals results in the return of viremia. Drugs targeting Vif would be expected to be evaded by the development of resistance mutations, therefore careful consideration of the most efficacious drug regimen will be necessary. An anti-Vif drug introduced into current ART regimens may be effective at reducing the burden and eventually eradicate the virus as it slowly emerges from viral reservoirs. A rather ominous alternative, however, would be the development of sufficient resistance mutations to allow viral escape. Concern has been expressed in the potential of APOBEC3G and 3F mutations conferring HIV-1 drug resistance (Mulder, Harari et al. 2008). An alternative therapeutic approach would be to administer three separate anti-Vif drugs, allowing APOBEC restriction to reduce the viral burden in lieu of using antiretrovirals that suppress replication. The use of multiple drugs to target Vif reduces the risk of escape mutants developing, as the virus would be required to develop numerous escape mutations prior to lethal hypermutation occurring by

APOBEC. The interactions between Vif, APOBEC3G, APOBEC3F, and the ubitiquin ligase components provide a number of potential target sites for inhibiting Vif function. The effect of inhibiting of these interaction sites need to be assessed both *in vitro* and *in vivo* to identify optimal drug targets.

Based on my experimental evidence, it is clear that humanized mice could serve as an excellent system to test *in vivo* the effects of Vif inhibitors. In addition, also based on my experimental evidence it is clear that Vif inhibitors are likely to have a dramatic effect on the ability of HIV to survive in infected patients perhaps ultimately yielding a cure for AIDS.

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