MECHANISMS OF HIV-1-MEDIATED CD4+ T CELL DEPLETION IN LYMPHOID TISSUE

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ABSTRACT

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Mechanisms of HIV-1-mediated CD4+ T cell Depletion in Lymphoid Tissue
(Under the direction of Dr. Lishan Su)

In this dissertation, I investigate the methods employed by HIV-1 to cause depletion of CD4+ cells within the lymphoid organ. Human *ex-vivo* tissue models for infection provide a relevant microenvironment for studying acute infection and subsequent pathogenesis, with only the influence of an innate immune response. Two viral isolates obtained from a rapid progressor patient with significantly different pathogenic phenotypes have been used to identify mechanisms of HIV-1-mediated cell death. The two isolates have significant sequence homology, particularly in the envelope (Env) region. However, isolate R3A demonstrates enhanced fusion as well as enhanced pathogenesis in both relevant *in-vivo* and *ex-vivo* organ model systems when compared to isolate R3B. Interestingly, when fusion is inhibited, R3A demonstrates pathogenic abilities through a bystander killing method as well.

Analysis of Env proteins both *in vitro* and *ex vivo* demonstrates that enhanced Env-mediated fusion (determined by the C-terminal Heptad Repeat off gp41) contributes significantly to the distinct pathogenicity observed by the pathogenic R3A Env isolate, while CXCR4-binding affinity does not correlate with pathogenicity. In the absence of Env-mediated fusion, however, it is also observed that the pathogenic HIV Env continues to deplete cells. Further analysis of these findings demonstrated the role of the HIV Env V1/V2 domain upon activation of plasmacytoid dendritic cells (presumably due to

enhanced CD4 binding affinity), which then induce bystander killing of uninfected lymphocytes.

Finally, I examine the role that the HIV-1 Nef protein plays (in concert with an Env of high CD4 binding affinity) to activate the host innate immune response, which likely contributes to the observed bystander cell death phenotype. This analysis suggests that the Nef protein may enhance HIV Env expression on the surface of virus-producing cells and hence on budded virions, which can then more robustly activate pDCs.

The findings from these studies aim to elucidate the mechanisms of pathogenicity utilized by a particularly pathogenic HIV Env isolated from a rapid progressor patient. These mechanisms shed light upon the nature of pathogenic viruses in total, and will hopefully aid in the development of therapy options for HIV-infected patients in the future.

This work is dedicated to both my family and friends, who have seen me through the particularly rocky roads I have traveled on over these past few years. There is nothing like true adversity to show you who and what really matters in life.

If you fall off a bike, the best thing you can do is get right back on.

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Introduction

The Current Blight of HIV-1

Human Immunodeficiency Virus type 1(HIV-1) is the causative agent for Acquired Immunodeficiency Syndrome (AIDS), and is a retrovirus that currently infects 42 million individuals worldwide(3). Since the disease's description in 1981, more than 25 million people have died(74, 94). Even more alarming is the current rate of new infections, which is continuously on the rise. In 2006, 4.3 million people were newly infected while 2.9 million died of AIDS(3, 74). This disease can truly be known as a pandemic in that it is present on every continent of the planet, with a marked increase between 2004-2006 observed in East Asia, Eastern Europe and Central Asia. Sub-Saharan Africa carries the largest toll of this pandemic, with 63% of total HIV-positive children and adults residing in this continent and a large fraction of these people living in South Africa. In fact, 34% of all deaths due to AIDS in 2006 occurred in South Africa, with 72% of total AIDS-related death occurring in Sub-Saharan Africa(3, 74). This clearly demonstrates the devastation that is caused by this disease. Unlike most infectious disease pathogens that primarily affect the poor and marginalized, HIV-1 infection is a plight that afflicts people of all races, economic groups and sexual preferences.

Initially deemed as the "gay plague" due to the misconception that the disease only affected the homosexual populations (as well as IV drug users), epidemiologic evidence soon indicated that bodily fluids transferred the infectious agent causing AIDS(40). In 1983, the CDC documented the first recorded heterosexual transmission, thus dispelling the belief that AIDS was strictly a "gay disease". Initial cases were confirmed in cities such as New York

and San Francisco, but soon after, cases were confirmed in Europe and in Africa. In a relatively short period of time of three years, the causative agent for AIDS (HIV-1) was isolated by initially by Montagnier and colleagues (Pasteur Institute) and then Gallo and colleagues (NIH), and formally designated as "Human Immunodeficiency Virus" by the International Committee of the Taxonomy of Viruses by 1986(18). A related, but immunologically distinct retrovirus, known as HIV-2, was discovered in 1986. HIV-1 can be further phylogenetically classified into three groups: M, O and N, with the M group consisting of 95% of global viral isolates(2). In 2008, the world recognized that 25 years had gone by since the first observation of HIV, yet much work is needed towards development of better therapeutics and a functional vaccine.

Characterization of HIV-1 Infection

The HIV-1 virion carries an enveloped structure with two copies of the RNA genome encased inside. HIV-1 infection requires either mucosal transmission (through sexual intercourse) or blood-borne transmission to successfully enter a host. The virus's primary target is the CD4+ lymphocyte, but it also infects other cells of the immune system such as macrophages and dendritic cells. Viral infection requires the viral envelope protein (gp120) binding to its primary cellular receptor, CD4. On lymphocytes, CD4 binding induces a conformational change with in the HIV envelope protein that allow for co-receptor binding. The co-receptor the virus uses is typically CCR5 or CXCR4, although other co-receptors can also be used under certain experimental conditions(108, 109).

Unlike some enveloped viruses that utilize a receptor-mediated endocytosis to gain entry into the host cell, HIV-1 (like most other retroviruses) directly fuses with the plasma

membrane of the susceptible cell. Upon gp120 binding to both receptor and co-receptor on a CD4+ T lymphocyte, another conformational change occurs within the envelope protein that allows presentation of the gp41 fusion protein to fuse to the cell. The transmembrane protein gp41 acts as a class I viral fusion protein, thus allowing fusion to the host cell(1). Under the current model, the protein has at least three conformational states: pre-fusion native state, pre-hairpin intermediate state, and post-fusion hairpin state(15, 67, 101). During viral and target cell membrane fusion, the coiled-coil regions (heptad repeats) assume a trimer-of-hairpins structure, positioning the fusion peptide in close proximity to the C-terminal region of the gp41 ectodomain. The formation of this structure appears to drive apposition and subsequent fusion of viral and target cell membranes. Membrane fusion leads to delivery of the nucleocapsid (containing the HIV-1 RNA) into the cytoplasm.

Upon entry into the cytoplasm, a partial uncoating of the virion occurs. Reverse transcription takes place using the HIV-1 RNA as template to generate double-stranded DNA. This DNA then enters the host cell nucleus through a nuclear pore, where integration occurs into the host chromosomal DNA. This integration step was initially thought to occur randomly, but it is now known to occur at sites of active gene transcription, which enhances the expression of the viral genome(85). The integrated viral DNA is transcribed into a single RNA, some of which is spliced. This RNA is then translated in the cell cytoplasm. Proviruses have been shown to first express regulatory proteins such as Tat, Rev and Nef; structural and enzymatic proteins are then produced to aid in viral assembly(1). The regulatory proteins have demonstrated a variety of functions, although much is still unknown. An immature virion characteristically buds from the membrane, where subsequent proteolytic cleavage occurs to produce mature virion. Also released at this time is free

gp120, which will be discussed later. Released virion generates an initial burst of replication (denoted as acute infection) but ultimately encounters the host immune response, which significantly lowers (but does not completely control) viral titer. This lack of virologic control leads to the chronic disease phase, which can persist for several years. The onset of AIDS progression is manifested by a decrease in circulating CD4+ T cells, falling below 200 cells/mm^3 [82]. Once a patient's CD4+ T cell count falls below this level, they become vulnerable to a host of opportunistic infections and various malignancies, yielding high morbidity and mortality.

Although infection of CD4+ lymphocytes has been of significant concern due to their being the primary target for HIV infection and depletion, research has clearly demonstrated that other cell types are infected or are engaged by the virion through receptor-mediated interactions. Cells of the myeloid lineage including monocytes, macrophages and dendritic cells have been shown to be significant facilitators of viral latency and persistence, due to their longer life spans in the host as well as their migration to lymphatic tissues upon infection. Monocytes differentiate into macrophages, where MHC II expression and B7 molecules are up-regulated and provide function as antigen presenting cells. Macrophages express receptors for HIV infection such as CD4 and coreceptors, and it is yet unclear what percentage of cells are infected or engage virion (and virus-infected cells) by phagocytosis. Although circulating monocytes are infectable *in vitro* and are found infected in seropositive individuals, macrophages are the most commonly infected cells found *in vivo* in addition to CD4+ lymphocytes(80).

Human dendritic cells (DCs) can be further divided into several populations that exist in the periphery and in lymphatic tissue: monocyte-derived dendritic cells (mDCs) and

plasmacytoid dendritic cells (pDCs). mDCs exist primarily in the skin and mucosal tissues. pDCs exist primarily in the blood and lymphatic tissue. Both populations exist in the periphery, albeit in different numbers. mDCs constitute 0.5% of peripheral blood, and play a significant APC role in the periphery(80). Plasmacytoid DC exist in much smaller numbers (0.1%), but are known as effector cells for innate immunity as well as providing APC function (albeit weakly compared to mDC)(88). Upon antigen encounter, both populations mature and migrate to regional draining lymph nodes. Many studies have shown that the numbers of both DC populations are decreased in blood during HIV Infection, although it is now thought that this decrease is both due to depletion as well as migration to lymph nodes.

pDCs provide the first line of defense in fighting against viruses and are shown to bridge innate immunity with the adaptive immunity against viral infection. A key hallmark of pDCs as immune effector cells is that they rapidly produce type 1 IFN upon exposure to viral infection. Compared with mDC, pDC express high levels of CD4, CD123 (or IL3Ra) and a few pDC-specific receptors including BDCA2, BDCA4 and ILT7(19). Most importantly, TLR7 and TLR9 are preferentially expressed in the endosome of pDC, endowing them as the professional sensors of viral RNA and DNA, respectively(51, 63). Upon exposure to viral RNA or DNA in the endosome, pDC are rapidly activated to produce immense amounts of type 1 IFN and IL6/TNFα(19, 70). It has been reported that viral RNA (or DNA) binds to TLR7 (or TLR9) to initiate a cascade of signaling events to activate the MyD88-IRAK-TRAF-IRF7 complex(19, 70). Activated IRF7 migrates into the nucleus to induce expression of IFN genes. Interestingly, HIV Env interacts with CD4, CXCR4/CCR5 and BDCA2, with unclear effect on pDC activation. CD4 binding is proposed to trigger endocytosis to expose HIV genome to TLR in the endosome (8, 34, 70). However, the effect

of Env binding to each of these receptors (CD4, CXCR4 or CCR5) on pDC activity is not clear.

As macrophages, mDCs and pDCs all have important APC and immuno-modulatory effects, infection of these cells may contribute significantly to the observed pathogenesis of HIV-1. It is thought that these cells may carry "latent reservoirs" of HIV which contribute to reactivation of virus when anti-retroviral therapy is halted in infected patients(6, 20). Further, upon initial infection in mucosal regions, the cells may be the first to interact with free virus and spread into the host lymphoid tissues. Interestingly, DCs may also play another pathogenic role due to interaction with HIV-1, which will be discussed later.

Models of HIV-1 Infection

Research into the pathogenic mechanisms of HIV-1 is significantly hindered by the lack of a suitable model system. HIV-1 is a human pathogen that infects no other host in a comparable way. A related virus such as Simian Immunodeficiency Virus (SIV) can model aspects of the infection and pathogenesis, but the models carry caveats with them. Namely, using primates as an infection model is both costly and difficult, causing them to be a limited resource for experimentation. Regardless, much has been learned from study of SIV infection in primate hosts. Specifically, SIV infection of Rhesus Macaques leads to a similar pathogenesis as observed in humans, characterized by general immune activation and progressive CD4+ T cell depletion. The Sooty Mangabee, however, is the natural host for SIV and demonstrates a homeostasis with virus during infection. This is characterized by a lack of immune activation, no CD4+ T cell depletion and ultimately no progression to AIDS, despite high levels of viral replication(27, 87).

Though studying SIV infection in primate models can provide much insight, study of HIV-specific infection and disease progression is still problematic. Developing a detailed study of HIV-induced pathology in lymphoid organs require analysis of tissue that carry human macrophages, dendritic cells and stromal tissue as well as human CD4+ T cells. For this purpose, our group and others have previously employed to use of the "SCID-hu Thy/Liv mouse" model for analysis of HIV-mediated pathogenesis. This mouse model requires the engraftment of human fetal liver and thymic tissue into immunodeficient C.B-17 scid/scid (SCID) mice(69, 75). The mice have been extensively characterized to repopulate with normal cell populations that promote long term T cell differentiation into predominantly resting cells of expected proportions(60). Further, this model has been shown to be permissive for infection with primary HIV-1 isolates, while refractory to tissue culture-adapted strains(28, 72, 92, 93, 95). Thymocyte depletion is observed during infection preferentially with syncytium-inducing (SI) CXCR4 tropic HIV-1 strains isolated from AIDS patients, but not with pre-AIDS isolates with NSI-/CCR5-tropic HIV isolates.

To further facilitate study of questions regarding pathogenic capabilities of HIV isolates, our group and others have also utilized the human fetal thymus organ culture (HF-TOC) system as a model that exhibits similar infection and pathology to that of the SCID/Hu Thy/Liv mouse. The HF-TOC system in an *ex vivo* model for HIV infection, that provides the benefit of ease of use for addition of experimental pharmacological treatments, over a window of 14 days. The human fetal thymus from a single donor can be dissected into relatively numerous fragments of equivalent sizes that retain thymic structure and composition, thus providing an excellent model system for HIV-1 pathogenesis study.

Both the SCID/hu Thy/Liv mouse model and HF-TOC system present excellent systems for study of HIV-1 infection, replication and pathogenesis in primary lymphoid tissue, in the face of only the innate immune system. As such, these models can be construed as models of acute infection of lymphoid tissue, where the adaptive immune system has yet to respond to insult.

Recently, our group and other have employed the use of the "humanized mouse" model for study of HIV infection(25, 55, 107). First described in 2004, this novel approach involved the transplant of CD34+ progenitor cells into the liver of new-born immuno-deficient mice (Rag2-/gammaC-/-)(16, 17, 96). As these animals lack endogenous B, T and NK cells, repopulation from the transplanted cells increases splenic, thymic, and lymph node populations to relevant proportions. Further, both CCR5 and CXCR4 are detected on human immature and mature T cells. HIV-1 infection and T cell depletion was observed in these mice, and T cells were recently demonstrated to be infected at mucosal sites (9, 55, 107). Interestingly, a similar mouse model has been recently described to carry both cellular and humoral adaptive immune responses against HIV, demonstrating that the humanized mouse model may be an exciting system for discerning both pathogenicity and immune protection against HIV infection (12).

Pathogenic proteins of HIV-1: Focus on Env

The focus of this study involves discerning aspects of the virus that significantly contribute to HIV-1 pathogenesis. Though HIV-1 is a relatively small virus, many viral proteins have been shown to influence direct and indirect cell death, in various cell types. It is hypothesized that HIV-1 regulates cell death in a way that is beneficial for itself in terms

of the requirements for its own life cycle, such as steps involving viral replication and transmission. In various studies, Tat, Vpr, Nef, protease and Env were all shown to contribute to the depletion of CD4+ T cells observed during HIV-1 infection.

Tat

Tat is one of the earliest protein expressed post infection, and both intracellular and soluble forms can be detected within HIV-1-infected patients. Ectopic expression of Tat induces apoptosis in uninfected cells through a caspase-8 and Fas-L dependent pathway(32, 65, 79). Various studies have also demonstrated a role for Tat in neuronal apoptosis and gliosis. However, endogenous Tat expression has shown anti-apoptotic effects in some systems through increased Bcl-2 expression and inhibition of antigen-stimulated proliferation of infected lymphocytes, which would typically lead to cell death(98).

Vpr

Vpr is an accessory protein that has been shown to be dispensable to viral replication in CD4+ T cells, although it has been shown to play a variety of pro-apoptotic roles. Soluble Vpr is cell permeable, and induces caspase-independent apoptosis in a variety of cell lines as well as release of Cytochrome C (a potent apoptotic factor)(53, 90, 104). Several groups believe that Vpr may play a dominant role in depletion of both infected and uninfected T cells in infected patients as well as causing G2 arrest in cell cycle progression (90,104). Vpr has also been shown to up-regulate LTR transcription, cellular activation and differentiation(7). These conflicting findings likely indicate both pro-apoptotic and anti-apoptotic roles of the protein.

Protease

Protease has been shown to activate caspase-8, and it also cleaves the anti-apoptotic protein Bcl-2, thus enhancing the direction towards an apoptotic pathway(76, 91). Together, these finding suggest that Protease may play a significant role in the death pathways of infected cells.

Nef

The exact role that Nef plays on cell death remains in question, although the proteins' necessary role in HIV-1-mediated pathogenesis strongly suggests some involvement in cell death. Much focus has been placed on the possibility of a Nef-deleted attenuated vaccine, due to the occurrence of a number of Long Term Non-Progressors (LTNPs) that carry viruses with Nef deletions(35). Whether Nef functions to enhance viral replication or to provide a direct pathogenic effect remains unclear. It has been demonstrated that cells stably expressing Nef have enhanced up-regulation of Fas-L as well as playing a role in the reduction of Bcl-2 expression(4). However, the fact that Nef significantly down-regulates CD4 would suggest the Nef protects the infected cell from super-infection by other virions, possibly prolonging the life of that cell for viral replication purposes(5, 36). It is also known that Nef mediates MHC I down-regulation to assist in further evading the host immune response and increase viral infectivity, as well as inducing Programmed Death 1 (PD-1) expression and thus inactivating cell proliferation and cytokine production(59, 73, 84). These functions may also be necessary to robustly express Env on the surface of budding virion. Thus, Nef and Env may work together to protect infected cells while inducing pathogenesis on un-infected cells.

Focus on the Env:

HIV-1 Envelope provides the first functional interaction between virion and host cell, and is the protein responsible for cellular tropism as well as immune evasion. The selection pressure placed on Env is very high because of these factors, and as such, genetic variability is the highest compared to other viral proteins. This variability occurs within the complex protein structure, but is focused on specific domains that will be further discussed.

The Env protein has been shown to induce cell death in infected and uninfected CD4+ T cells by several mechanisms. Direct lysis of cells can be Env-mediated through membrane fusion(61, 62). Fusion-induced syncytia (multi-nucleated cell) formation will lead to lysis or apoptosis. Further, soluble gp120 is implicated in bystander uninfected cell death through various means. Binding of CD4 and co-receptors are implicated as is through induction of cytokines that participate in maintaining the state of chronic activation observed in patients(49). Because Env may contribute the most significantly to both infected and uninfected cell death by a variety of methods, we will herein focus our study on structural aspects of Env that may confer the observed pathogenic phenotype.

The Env protein is translated from the Golgi as a glycoslyated polyprotein precursor (gp160), but is then proteolytically cleaved by cellular enzymes to mature gp120 and gp41(2). Post cleavage, the two glycoproteins form a non-covalent association and the proteins are shuttled to the cell surface. The gp120 comprises the surface subunit and the gp41 serves as the transmembrane protein(2). This association is fairly weak and contributes to the substantial amount of gp120 shed from the infected cell surface. These Env proteins are found to exist as trimers on the cell (and virion) surface, which aids in the binding and fusion process necessary for viral entry(1). The gp120 protein functions to bind CD4 and co-receptor, as well as play roles in immune evasion, while the gp41 protein serves as the

transmembrane anchor that tethers the Env protein to the cellular (and virologic) membrane. The gp41 is also required to form the fusion complex, allowing for viral entry into the host cell.

Gp120 can be divided into five variable (V) and five constant (C) regions, and the protein structure is highly glycosylated(1). This glycosylation is so significant, in fact, that it provides a "sugar-cloud" obscuring much of gp120 from immune detection. The C regions are typically quite conserved between isolates, and link the V loops together. Clearly, the loops comprise most of the variability seen between isolates, as they are the domains that interact with the host cell for viral entry. Gp120 can be further divided into an inner domain and an outer domain that are connected by a bridging sheet(77). When the Env protein interacts with a host cell, the CD4 receptor binds to the groove of the gp120 at the junction of the bridging sheet, inner domain and outer domain (which forms the CD4 binding pocket)(78). CD4 residues that come into contact with gp120 tend to be found in a tight cluster, whereas the gp120 domains involved in binding are spread through several regions of Env, due to the complexity of the Env proteins structure. A majority of Envs isolated from tissue culture adapted strains demonstrate very open conformations with exposes CD4 binding sites, presumably due to the lack of immune pressure in cell culture. This may be the reason why tissue culture adapted strains have enhanced "CD4-binding affinity", as compared to primary isolates(52, 106).

Upon CD4 binding, the gp120 structure becomes more rigid with a significant loss of entropy(77). This stabilization provides for conformational shifts amongst the inner domain, thus providing access for co-receptor to its binding site. It is suggested that the V1 and V2 loops move away from the co-receptor binding site to remove an impediment from binding

while the V3 loop appears to move toward and aid in co-receptor binding(89). Binding of co-receptor is strongly mediated by the V3 loop, and provides yet another conformational shift to promote viral membrane-cell membrane fusion to occur(102). Essentially, the gp120 trimer facilitates the gp41 trimer to form a "six-helix bundle" of coiled-coils, comprised of the gp41 extracellular domains(66). This fusion "mouse trap" snaps shut allowing for entry of nucleocapsid into the cell.

The gp41 protein can be divided into four general domains: the fusion peptide, extra-cellular (endodomain) domain, transmembrane domain, and intracellular (cytoplasmic) domain(66, 67, 100). The elements required for Viral Env-cell membrane fusion are carried within the extra-cellular domain and can be further divided into the N terminal and C terminal heptad repeats (N-HR and C-HR) and a cysteine bound mini-loop region in between that connects them.

This "six-helix bundle" that fuses to and enters the cellular membrane is known as the gp41 "core structure", and consists of three N-HR peptides that form a trimeric core and three C-HR peptides that bind to the grooves of the N-HR timer in an anti-parallel manner(66, 67). This formation of the "six-helix bundle" functions to bring the viral and cell membranes close in proximity to allow for a viral synapse to form and fusion to occur.

Due to the complexity of the Env structure and multiple steps necessary to function in coordination for viral Env-cell membrane fusion to occur, it is not surprising that alterations within many of gp120 V loops or gp41 domains can either impede fusion or enhance syncytia formation(13, 22). Env-cell fusion is observed to be a calculated maneuver, as enhanced stability of Env can induce more robust anti-HIV-1 antibody responses(13, 22,

105). As a result, it is likely that viral isolates that demonstrated enhanced fusogenicity have arisen in the absence of functioning immune responses in the infected host.

As mentioned earlier, the HIV-1 Env protein plays many reported roles in pathogenesis. In tissue culture experiments both immortalized and primary cells can exhibit syncytium formation that leads to cell death. Syncytium formation can occur due to infection, but also when uninfected cells are cultured with cells expressing HIV-1 Env on the cell surface, indicating that fusion itself can induce apoptosis. This pathway has been dubbed "Hemi-fusion" and is found to be caspase-3 dependent(37). Env-mediated fusion has also been demonstrated to be responsible for single-cell lysis of infected cells, as well as TNF-driven apoptosis in both lymphocytes and macrophages(44, 45). Apart from fusion, cells expressing Env (or virions) can also induce apoptosis in cells expressing CD4, through a Fas-dependent mechanism(4).

CD4+ T Cell Depletion by HIV-1

The preponderance of evidence suggests that lymphocyte depletion by HIV-1 occurs through several mechanisms. Furthermore, the fact that only roughly 1% of cells in the periphery of an infected individual are productively infected clearly demonstrates that robust uninfected cell depletion must occur during HIV-1 infection. Correlating with this, studies have demonstrated that only 0.01% of virions found both *in vitro* and *in vivo* during infections are found to be infectious. The lack of infectious virion and the significant immune depletion observed clearly suggests that non-infectious virions may play a role in uninfected (bystander) cell death.

HIV-1 Env has been implicated in multiple pathways of cell killing, for both infected and uninfected cell death. In various models of infection (mentioned earlier) studies have demonstrated a prominent role for both direct cell lysis (necrosis) and apoptosis as mechanisms for lymphocyte depletion by HIV-1. As the different pathways are discussed further, I will divide the methods into fusion-dependent versus fusion-independent killing of lymphocytes by HIV-1.

Fusion-Dependent Cell Death:

The general dogma surrounding HIV-1 interaction with T cells is that the Env of virion must fuse to the cell in order to orchestrate the hostile take over of host cell for infection. During productive infection, it may benefit the virus to protect the infected cell from death until the cellular machinery is successfully hijacked and progeny virion is produced. After viral egress, direct lysis or necrosis has been observed in infected cells *in vitro*. This is presumably due to a massive increase in the permeability of the cell surface due to budding of large amounts of virus. Another possibility suggested is that cell death is due to the accumulation of unintegrated viral DNA and subsequent interruption of cell function. Alternatively, cytopathic effect may be due to complexing of intracellular CD4 molecules with HIV-1 Env(50). The relative impact of direct cytopathic effects has been difficult to ascertain due to several reasons: while direct lysis is the predominant mechanism for cell death in cell culture with lab-adapted strains(11, 14, 61, 62), apoptosis may be the chief contributor to cell death of both infected and uninfected cells in *ex vivo* lymphoid culture systems(39, 41, 71).

We have previously shown that in a lymphoid model of infection, apoptosis of CD4+ cells increases in both p24+ and p24- cells due to HIV-1 infection, and this process is shown to be caspase-mediated. Further, inhibition of fusion (by C-HR peptide analog C34) can protect significant numbers of both cell populations from apoptotic death(71). This is important as inhibition of viral replication of HIV-1 during infection did not protect cells from apoptotic death, suggesting that fusion is more relevant than viral replication as a mediator of apoptosis. Interestingly, the induction of apoptosis was preferentially observed in tissue infected with a virus containing a highly fusogenic Env, versus the same virus carrying a less fusogenic Env, further supporting the idea the Env-mediated cell death is significantly fusion-dependent(71).

Addition of AT-2 inactivated virion to lymphoid tissue failed to recapitulate the pathogenesis observed by productive virus, suggesting that productive infection or cell-associated Env is essential for cell depletion. This agrees with other findings that cells that express HIV-1 Env (either through transfection(33, 81) or infection(10)) are specifically shown to induce apoptosis in neighboring uninfected cells, through a fusion-dependent pathway. These findings suggest that this apoptotic pathway involves classical mechanisms of apoptosis such as release of Cytochrome C and activation caspases 9 and 3, while not involving Fas/FasL signaling. These findings would culminate in a picture that suggests that during HIV-1 infection, a significant portion of both infected and uninfected cells undergo a caspase-mediated apoptosis that is viral Env-cell membrane fusion-dependent.

Autophagy

The catabolic process known as autophagy involves the degradation of cellular components through the lysosome, as a natural part of cell growth and developments(21). It

is known that this process is involved in degradation of damaged organelles, membranes and proteins, but recently it has been suggested as a process that can lead to both caspase-dependent apoptotic death and caspase-independent, non-apoptotic death(21).

Recently, the process of autophagic apoptosis has been observed to occur amongst uninfected cells during HIV-1 infection, and this process was found to be fusion-dependent(29, 31, 64). It was observed that blockade of autophagy through either drugs or siRNAs specific to autophagic genes completely inhibited the observed apoptosis. Although CD4 and co-receptor binding were found to be necessary to initiate gp41-mediated fusion, signaling through neither receptor was found to be necessary to induce the autophagic response. Further, inhibition of fusion (by T20 or C34) completely inhibits this pathway of cell death(24, 30).

It is not known whether autophagic activity in dying cells actually mediates death or whether it simply occurs as a process alongside the apoptotic pathway. In any event, various data suggest that Env-cell interactions of CD4, co-receptor and gp41 culminate in viral Env-cell membrane fusion, which demonstrates an important role in the apoptotic cell death of both infected and uninfected cells.

Fusion-Independent Cell Death:

As mentioned earlier, a relatively small number of productively infectious virions exist in the periphery of a (non-ART treated) chronically infected HIV-1 patient, yet immune cell depletion still occurs at a steady pace. With such little productive virus present, what contributes to fusion-independent cell death? A major culprit is seen with non-infectious viral particles such as soluble gp120, which are found to circulate in high numbers

throughout the periphery of an infected individual. It is thought that viral particles may significantly contribute to both the state of chronic immune activation as well as play roles in fusion-independent apoptosis.

Fusion-independent apoptosis of uninfected cell is thought to occur through several pathways. One method is termed Activation-Induced Cell Death (AICD), and occurs through repeated antigenic stimulation, culminating in a Fas/Fas-L mediated cell death(26, 54, 57). The role of Fas-mediated cell death has been a subject of much study, due to the fact that the proportion of Fas-expressing T cells in patients increases with disease progression, and Fas stimulation causes enhanced apoptosis of CD4+ T cells from HIV-1-infected individuals compared to uninfected controls(4). It is known that ligation of CD4 receptors will activate Fas expression on T cells *in vitro*, so it is believed that soluble gp120 (or Env-expressing cells) may constantly bind to CD4 on uninfected cells, thus activating Fas expression at a chronic level. This forms a plausible mechanism for how resting T cells may become chronically activated to undergo activation-induced apoptosis. However, conflicting evidence from freshly isolated PBMCs from HIV-1-infected patients demonstrated impaired Fas L expression compared to controls(56). This suggests that there may be an active suppression of Fas ligand expression in HIV-1 disease.

Other studies have suggested the possibility of co-receptor binding by non-infectious particles as a possible contributor to the chronic activation and bystander apoptosis observed. The co-receptors CXCR4 and CCR5 are both chemokine receptors that play cellular functions primarily related to chemotaxis of cells due to ligand expression. In a model proposed by Holm and Gabuzda, HIV-1 virions bind to CD4+ T cells, and induce signals through co-receptors. These signals both activate and induce apoptosis in previously

activated CD4+ T cells(48, 49). These findings go on to suggest the importance of host cell protein expression on the surface of the virion (MHCII and B7-2). These results provide a model for initial HIV-1 mediated activation for enhanced viral replication, but also an induction of apoptosis in bystander activated cells. This presents a conflict with studies by Denizot et al. that suggest chemokine binding but not signaling is required for apoptosis to occur(24), although discrepancies may be strictly due to models employed.

Finally, a relatively new mechanism is suggested that involves a host factor as a prime contributor to bystander apoptosis during HIV-1 infection. The TNF super-family has been studied at length for their role in HIV-1-mediated cell death(23, 45), but recent studies suggest that TRAIL (TNF-Related Apoptosis Inducing Ligand) has a significant involvement in bystander killing during HIV-1 infection. TRAIL has been shown to induce apoptosis in a variety of cells, due to virus infection or tumor progression(42, 83, 97, 99). Addition of exogenous TRAIL has induced a selective apoptosis of uninfected CD4+ T cells during HIV-1 infection of a human *in vitro* model system. Earlier studies demonstrated that Type 1 IFNs were the major inducer of TRAIL(58), and both monocytes and pDCs were shown to respond to HIV-1 by expressing TRAIL, either due to interaction with virion or Type 1 IFNs(43, 103). Further, monocytes were shown to produce both soluble TRAIL (sTRAIL) and membrane bound TRAIL (mTRAIL)(46).

TRAIL has two receptors, DR4 and DR5, which are activated to induce apoptosis. Interestingly, cells carry three more decoy receptors thought to inhibit TRAIL-induced death on normal cells. Herbeuval and colleagues observed that the death receptors increased on uninfected CD4+ T cells in direct correlation to viral load, indicating that a component of viral replication may play a distinct role in receptor expression on uninfected cells. As

mentioned earlier, HIV-1 replication produces tremendous amounts of non-infectious particles due to its error prone replication process. As of such, AT-2-inactivated virion were used to demonstrate that non-infectious viral particles were equally successful at inducing DR5 expression on the CD4+ (but not CD8+) T cell surface, thus implicating HIV-1 Env in this process(47).

These findings are both interesting and troubling, in face of IFNα therapy for HIV-1 and co-infection. Type 1 IFNs improve the survival of activated T cells, while having little to no effect on resting T cells(68). If Type 1 IFNs both prepare uninfected CD4+ T cells for TRAIL-mediated apoptosis as well as improving survival of cells primed for infection, then IFNα therapy may be severely detrimental to the infected host.

Recent analysis of newly HIV-1-infected patients demonstrate that levels of TRAIL have found to spike in the plasma of individuals during early acute infection, and this may be a significant contributor to resulting CD4+ T cell loss and ineffective immune responses(38). However, recent studies suggest that HIV-1 infection primes cells for TRAIL-mediated apoptosis, and would be targeted in recombinant TRAIL therapy. Particularly, HIV-1 viral burden was diminished during treatment suggesting that latent HIV-1 reservoirs were being depleted as well. Even though a level of bystander killing of uninfected CD4+ T cells may occur due to TRAIL expression in HIV-1-infected patients with unsuppressed viral replication, Badley and colleagues argue that recombinant TRAIL therapy may be very useful in ART-treated patients(86). Further analysis will have to be performed to determine whether recombinant TRAIL would be the appropriate therapy, or whether suppression of TRAIL-mediated apoptosis would be more beneficial.

Aim of Dissertation

During acute infection of both HIV-1 and SIV, it has been recently shown that CD4+ T lymphocytes are robustly and rapidly depleted in lymphoid tissues, and the partial recovery that ensues is gradually depleted during the chronic stage of disease. In both the acute and chronic stages of infection, the bulk of evidence demonstrates that direct infection does not significantly contribute to the observed depletion. Rather, the interaction between the host immune system with viral proteins as well as host proteins are found to significantly contribute the degree of immune activation and immune incompetence of the host. The various mechanisms that the envelope of HIV-1 utilizes to induce cell death remain to be fully elucidated. Studies addressing the functional domains of Env that contribute to this cell death may provide direction towards further therapeutic directions that can be used to protect the host.

In this dissertation, I will investigate the various mechanisms that HIV-1 Env utilizes to induce both fusion-mediated cell death as well as fusion-independent cell death. I will employ the human fetal thymus organ culture (HF-TOC) as an archetypal model for study of viral pathogenesis in lymphoid organs. As it has been previously demonstrated that this system is refractory to laboratory-derived viral isolates, HFTOC provides an excellent representative model for *in vivo* lymphoid tissue infection for study of clinically derived viral isolates. Using this model system to investigate Envs derived from primary isolates, and I plan to accomplish several tasks. First, I will utilize genetic manipulation of viral Envs to elucidate the domains that contribute to enhanced pathogenicity observed with a particularly pathogenic isolate. Further, I will present enhanced fusogenicity as a possible mechanism for enhanced pathogenicity of this isolate. Finally, I will genetically isolate the Env domains and

provide a mechanism for fusion-independent cell killing observed during HIV-1-infection of the lymphoid organ.

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Chapter 2

The Heptad Repeat (HR) 2 Domain Is the Major Determinant for Enhanced HIV-1 Fusion and Pathogenicity of A Highly Pathogenic HIV-1 Env.

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Abstract:

HIV-1 mediated depletion of CD4+ lymphocytes in an infected individual is the hallmark of progression to AIDS. However, the mechanism for this depletion remains unclear. To identify mechanisms of HIV-1 mediated CD4 T cell death, two similar viral isolates obtained from a rapid progressor patient with significantly different pathogenic phenotypes were studied. One isolate (R3A) demonstrates enhanced pathogenesis in both *in vivo* models and relevant *ex vivo* lymphoid organ model systems, when compared to isolate R3B. The pathogenic determinants were previously mapped to the V5-gp41 envelope region, correlating functionally with enhanced fusion activity and elevated CXCR4 binding affinity. To further elucidate specific differences between R3A and R3B within the V5-gp41 domains that enhance CD4 depletion, R3A-R3B chimeras studying the V5-gp41 region were developed. Our data demonstrates that six residues in the ectodomain of R3A provide the major determinant for both enhanced Env-Cell fusion and pathogenicity. Furthermore, three amino acid differences in the Heptad Repeat (HR) 2 domain of R3A determined its fusion activity, and significantly elevated its pathogenic activity. The chimeric viruses with enhanced fusion activity, but not elevated CXCR4 affinity, correlated with the high pathogenicity in thymus organ. We conclude that the functional domain of a highly pathogenic HIV-1 Env is determined by mutations in the HR2 region that contribute to enhanced fusion and CD4 T cell depletion.

Introduction:

Human Immunodeficiency virus Type 1 (HIV-1) is the causative agent for Acquired Immune Deficiency Syndrome (AIDS), which is characterized by a dramatic loss of CD4+ lymphocytes and impairment of the immune system against invading pathogens (13, 21, 22). Though much has been determined regarding interactions between HIV-1 virus and CD4+ target cells, the mechanisms by which the HIV-1 virus depletes CD4+ lymphocytes remain incompletely understood. Various studies have demonstrated that in an HIV infected host, both infected and uninfected cells are prone to destruction, albeit by different pathways (15, 18, 29). Recently our group and others have shown that while binding of CD4 and chemokine receptors contribute to syncytium formation in vitro, viral membrane fusion by the envelope glycoprotein plays an important role in depletion of both uninfected and infected cells by HIV-1 and SHIV *in vivo* (1, 11, 12, 26, 29).

HIV-1 entry into a cell is mediated by a multi-step process that begins with a high affinity binding between viral envelope (gp120) and the cellular CD4 receptor (9, 14, 16). This binding causes a conformational change in the viral envelope, allowing for subsequent co-receptor binding (mainly CCR5 or CXCR4). Upon co-receptor binding, another conformational change is thought to take place that allows gp41 to engage the cell to form a fusion complex. Envelope proteins have been demonstrated to exist as a trimer, allowing for three gp41s to form a fusion assembly through non-covalent interactions. This fusion assembly is determined to exist in a 6-helix bundle formation as the fusion event takes place, allowing for the virion to fuse to the host cell(5, 24).

The envelope glycoprotein (Env) of HIV plays a significant role in viral pathogenesis, as seen in several *in vitro* and *in vivo* models of infection. The Env functions to mediate viral entry of cells and is also a major target for immune responses (31, 39). Initially formed as a precursor protein (gp160), subsequent cleavage by a cellular protease yields the surface subunit (SU) gp120 and the transmembrane (TM) gp41, although the gp120 and gp41 interact non-covalently (36). The gp120 protein is comprised of five variable (V1-V5) and five conserved constant (C1-C5) domains, and binds CD4 and the co-receptors. The gp41 protein is comprised of an amino-terminal fusion domain and two heptad repeats (HR-1 and HR-2) in the ectodomain (extracellular domain), a single transmembrane domain, and a cytoplasmic tail (intracellular domain)(8, 10, 36, 37). Due to the discovery of fusion inhibitor peptides such as C34(23, 24) and T20 (38), much is now known about the fusion complex formed by the HIV-1 fusion domain. Similar to other viral envelopes that carry a type 1 fusion complex (such as influenza and corona viruses), the ectodomain of HIV-1 Env carries two heptad repeats that form a coiled-coiled structure. In order for HIV-cell fusion to occur, the HR-1 domains of the trimeric Env protein must interact with the cell surface. Following this initial interaction, HR-2 domains are thought to intertwine over the HR-1 coils to form a stable 6-helix bundle, which represents the gp41 core structure. X-ray crystallographic studies show that the 6-helix bundle core consists of the HR-1 and HR-2 peptides bound in an anti-parallel manner. This structure brings the fusion peptide to the target cell membrane, allowing for the formation of a fusion pore and the entry of virions into the cell.

HIV-1 Env expressed on the surface of infected cells can induce cell-cell fusion with adjacent uninfected cells to form multinucleated syncytia and single cell lysis in cell culture,

and apoptosis in primary cells. Various models (both *ex vivo* and *in vivo*) have been utilized to study HIV-1 induced depletion of CD4+ lymphocytes. Models such as SCID-hu thymus-liver, tonsil histoculture, and human fetal thymus organ culture (HF-TOC) have demonstrated significant use in the study of acute infection and pathogenesis in the appropriate lymphoid organ microenvironment, as they retain the organ structure and do not require exogenous stimulation for productive viral infection to occur (2, 20, 28, 32). More importantly, tissue culture-adapted HIV-1 isolates such as HXB2 fail to replicate in the SCID-hu Thy/Liv or HF-TOC models (30, 33). Organ models such as the SCID-hu Thy/Liv and HFTOC thus more accurately demonstrate infection, replication and pathogenicity of primary HIV-1 strains.

Here, HF-TOC is used to investigate mechanisms by which an HIV-1 virus with a highly pathogenic viral Env is able to deplete CD4+ lymphocytes. Two viral isolates obtained from rapid progressor patient 3 of the ALIVE cohort (40) show significant sequence homology, particularly in the Env region, while they carry stark differences in pathogenic ability (26, 27). One isolate (denoted as R3A) was found to demonstrate enhanced fusion in cell-cell fusion assays as well as enhanced pathogenesis in relevant ex-vivo/in vivo organ model systems, when compared to isolate R3B. To define the pathogenic determinants that differentiate R3A from R3B, this study demonstrates that enhanced fusogenicity of R3A (governed by the ectodomain of the gp41), but not the elevated CXCR4 binding affinity, confers the pathogenic phenotype in HFTOC. We further demonstrate that three amino acid differences in the HR-2 domain allow for this enhanced fusion for R3A Env, defining a possible mechanism for a pathogenic HIV-1 envelope.

Materials and Methods:

Cells: A293T and Magi-CXCR4 cells (obtained from the NIH AIDS Research and Reference Reagent Program) were cultured in Dulbecco's Modified Eagle medium supplemented with 10% (vol/vol) bovine calf serum (Sigma Chemical, St. Louis, Mo.) and 100 μg of penicillin and streptomycin/ml. SupT1 and BC7 cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Sup-T1 from Dr. James Hoxie and NIH: BC7 (Cat# 11434), from Dr. James Hoxie) were cultured in RPMI 1640 supplemented with 10% (vol/vol) bovine calf serum and 100 ug penicillin and streptomycin/ml. HOS-CXCR4 cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (HOS-CD4-Fusin) from Dr. Nathaniel Landau(3)) were stably transfected to express LTR-driven luciferase and were cultured in DMEM supplemented with 10% (vol/vol) bovine calf serum and 100 μg penicillin and streptomycin/ml. All viral isolates were prepared and stored in Iscove's Modified Dulbeccos Media supplemented with 10% (vol/vol) bovine calf serum and 100 μg penicillin and streptomycin/ml. Peripheral blood monocytes were purified from the blood of healthy HIV-1 negative donors by Ficoll-Plaque density gradient centrifugation and cultured in Iscoves Modified Dulbeccos Media supplemented with 10% bovine calf serum and 100 μg penicillin and streptomycin/ml. PBMCs were stimulated with 5 μg phytohemagglutinin (Sigma)/ml and 20 units/ml rIL-2 (Sigma) for 3 days, and then cultured in 20 units/ml rIL-2.

Viral Isolates and drugs: Isolation of Env from primary isolate and cloning of NL4-R3A and NL4 R3B have been previously described (27). Peptides C34 were synthesized by at the

MicroChemistry Laboratory of the New York Blood Center (Kindly provided by Dr. S. Jiang). C34 was reconstituted in phosphate buffered saline at a concentration of 1 mg/ml.

Chimeric Envelope construction: R3A and R3B Envelope chimeras were developed in a retroviral vector plasmid (HSPG (6, 27)) using an over-lap PCR strategy with the following primers**: V3F-** GTAACTCTAGGACCAGGCAGAG, **V5F-** GCTGTGTTCCTTGGGTTCTTGG, **V5R-** CCAAGAACCCAAGGAACACAGC, **gp41F-** CACCATTATCGTTCCAGACCCG, **gp41R-** CGGGTCTGGAACGATAATGGTG and **HSPGR-** CTAAAGCGCATGCTCCAGACTG. Chimeric Env regions were cloned back into the HSPG vector expressing Env, and verified by both restriction digestion and sequence analysis. The half-virus strategy was utilized to clone the chimeric Envs into the full-length NL4.3 backbone, and progeny virus was developed by transfection into A293T cells. Site-directed mutagenesis was performed to create single amino acid changes in the ectodomain of R3A, and was verified by sequence analysis.

Viral Production: VSVg-pseudotyped retrovirus was produced by calcium phosphate co-transfection of A293T cells with VSVg, Gag/Pol, and HSPG retroviral DNA as previously described(4, 27). The HSPG retroviral construct contains GFP under the control of the phospho-glucose kinase promoter (after transfection) or the MSCV LTR (after transduction). Viral supernatant was harvested at 48 and 72 hrs post transfection, clarified by low speed centrifugation, aliquoted and frozen at -70, as previously mentioned. Infectious virus for R3A, and R3B chimeras were derived as follows: chimeric Envs were cloned into p83.10 plasmid containing partial NL4 genome (EcoRI to XhoI) and co-transfected into A293T cells with the p83.2 plasmid containing the remaining NL4.3 genome. Supernatant was harvested at 48 hrs post transfection, and co-cultured with stimulated PBMCs. Pseudotyped NL4-Luciferase viruses (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3.Luc.R–E– from Dr. Nathaniel Landau(7,

17)) expressing chimera Envs were generated by co-transfection of pNL4-Luc and HSPG-Envs into A293T cells as mentioned previously. Viral supernatants were harvested daily and were tittered for expression of Gag by p24 ELISA.

Viral quantitation: Gag was detected in viral stocks as well as supernatant from HFTOC infections using a p24 enzyme-linked immunosorbent assay (ELISA) kit (AIDS Vaccine Program, NIH). Viruses were quantified for infectious units/ml by infection of Magi (CXCR4) cells, as we have reported(27, 30, 33).

Human Fetal Thymus Organ Culture (HFTOC): The procedure for infection and culture of human fetal thymus has been previously described (26-28). In brief, human fetal thymuses (19 – 22 weeks of gestation) were dissected into ~ $2mm^2$ fragments, using a dissecting microscope to retain the lobe structure for each fragment.

Four fragments were placed on organotypic culture membranes (Millipore) and under-laid with HFTOC media (previously described) were plated in a 6 well tissue culture plates. Thymic fragments were infected with equivalent amounts of virus (100-800 IU) in 15 ul per fragment. Viral and mock supernatants were derived from the same donor sample per experiment.

Thymic fragments were cultured at 37°C in 5% $C0_2$ for the length of each experiment. At harvest day of experiments, thymic fragments were disassociated in 350ul PBS with 2% FBS using prestles (Bellco Co.) for Florescent-Activated Cell Sorting (FACS) Analysis.

Florescent Activated Cell Sorting (FACS) Analysis: Thymocytes (and other cells) were stained with CD4-PE and CD8-FITC antibodies (Caltag) for surface staining. Cell viability

was assessed using staining for 7AAD. Analysis was performed using Summit Software, as previously reported.

Virus-Cell Fusion Analysis: NL4-Luc pseudotyped virus expressing R3A, R3B and chimera Envs were generated by transfection of 293T cells. Viruses were titered by Magi Assay, and SupT1 cells were infected by spinoculation and then cultured for 48hrs. At endpoint, cells were harvested and lysed for luciferase analysis. Luciferase was measured using luciferase assay buffer reagents (Promega) and a Fluorstar luminometer.

Cell Fusion Analysis: BC7 cells were transduced with retrovirus to express HIV Env, which was assessed for relative expression by FACS analysis by 2G12 staining (10μg/ml, obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody (2G12) from Dr. Hermann Katinger). Cells were then co-cultured with HOS/LTR-luc cells for 48hrs, at which cells were pelleted and lysed for luciferase activity. To measure the rate of fusion of various Env-expressing BC7 cells to HOS LTR-luc cells, C34 was added to wells (10μg/ml) to inhibit fusion at various time points, and then pelleted and lysed at 48hrs for luciferase activity.

Statistical Analysis: Both Student's T Test and nonparametric Mann –Whitney tests were performed to determine statistical significance of data. All analysis was performed using Graph Pad Prism software.

Results:

The enhanced pathogenicity of R3A envelope is determined by mutations in the gp41 Ectodomain.

We have previously demonstrated that the R3A Env is significantly more pathogenic than R3B Env in the thymus organ models. The enhanced pathogenicity of R3A Env is correlated with high CXCR4 binding and more fusogenic activity than R3B (26-28). Specifically, chimera viruses containing the V5 and gp41 domains of R3A in the R3B envelope background demonstrated an elevated pathogenic phenotype of CD4+ lymphocyte depletion, when compared to the parental NL4-R3B virus. Five differences exist in the V5 domain between R3A and R3B envelope, while ten amino acid differences exist in the gp41 region, with six differences in the ectodomain and four differences in the cytoplasmic tail of the R3A Env. In order to determine which amino acid differences in these domains of R3A envelope contribute to pathogenesis, chimera Envs between R3A and R3B were created (Fig. 1a). One chimera (denoted as R3A/B gp41) carried the entire gp120 of R3A with the R3B gp41. The second chimera (R3A/B cytoplasmic tail) retained the R3A gp120 and the ectodomain of gp41, while carrying the gp41 cytoplasmic tail of R3B. The third chimera (R3B/A Ectodomain) carried the R3A ectodomain in the R3B Env. Upon transfection of Env expressing plasmids into A293T cells, Western blot analysis was performed to show that all HIV-1 recombinant Env were similarly expressed and processed (data not shown). To further show surface expression of HIV Envs, surface expression on A293T cells were assessed using FACS analysis. Similar surface levels of all recombinant Env genes were detected by 2G12 antibody staining (Fig 1b), which binds the glycolsylated gp120 (34).

To determine whether specific regions of the R3A play a role in CD4+ cell depletion, recombinant HIV-1 with the R3A/R3B chimeric Envs in NL4.3 backbone were produced and studied. Recombinant viruses have similar replication and CD4 depletion in PHA-activated PBL *in vitro*. Infection of activated PBMCs demonstrated that the viruses were equivalently infectious, as assessed by p24 gag protein (data not shown). We found that there was no difference in viral production between the recombinant viruses as we have previously reported with R3A and R3B in activated PBMC (28). CD4+ lymphocyte depletion was assessed by FACS analysis over several time points, and depletion was also found to be equivalent by parental strains and chimeras over time (Fig 1c).

R3A shows elevated pathogenic activity in human lymphoid organs, which has been mapped in the gp120 V5 and the gp41 regions (28). In order to further dissect the determinants within the V5-gp41 that impact CD4+ cell depletion by R3A Env, the R3A/R3B chimera viruses were used to infect human fetal thymus fragments (HFTOC). At various time points after infection, viral p24 level in the HFTOC supernatant was harvested and viral replication was measured using ELISA for p24 antigen. Virus replication over 11 days of HFTOC infection was found to be different (Fig 2a), in that NL4-R3A and viruses possessing the R3A gp41 ectodomain replicated to higher levels than NL4-R3B and viruses carrying the R3B gp41 ectodomain (R3A/B gp41). On day 11 post infection, fragments were harvested and thymocytes cells were counted and analyzed by FACS analysis. Upon infection of HFTOC, a significant difference in CD4+ thymocyte depletion between R3A and R3B was detected (Fig 2b). Thus NL4-R3A depleted CD4+ lymphocytes by 80%, while NL4-R3B depleted cells by only 40%. The relative depletion of CD4+ thymocytes by NL4-R3A/Bgp41 was significantly lower than NL4-R3A, but not different from NL4-R3B. Both

the chimera NL4-R3A/B-cytoplasmic tail and R3B/A ectodomain depleted CD4+ cells to levels similar to NL4-R3A, and were both significantly more pathogenic than NL4-R3B. Therefore, enhanced replication and CD4+ thymocyte depletion of R3A in the HFTOC model were determined by the six amino acid differences between R3A and R3B in the ectodomain of gp41.

The second Heptad Repeat (HR-2) of R3A gp41 determines the enhanced fusion ability of R3A.

The ectodomain of HIV-1 gp41 encodes the fusion domain and two heptad repeats, thereby allowing the virus to enter the host cell (5, 24, 25). It has been previously demonstrated R3A Env confers enhanced fusion ability to cells, compared to R3B Env (26, 27). The R3A and R3B ENV genes encode the same fusion peptide in gp41. Thus other determinants in the ectodomain of R3A Env must be important. The ectodomain also contains the N terminal heptad repeat (HR-1), a mini-loop region, and the C-terminal heptad repeat (HR-2). From sequence analysis (using HXB2 for consensus) (28), two amino acid changes (Q543R and Q564H) exist in the HR-1, three exist in HR-2 (S644N, N651I and E665K), and one in the cysteine mini-loop region between the two HR domains (H620D). It is possible that changes in an ectodomain could either alter the interaction between the HR-1 domain with the HR-2 domain, or with interactions between the HR-1 domains in the coil-coil formation, leading to either enhanced or reduced fusion. A modeling diagram was constructed over the known crystal structure for the ectodomain of HIV-1 gp41(5) carrying the individual amino acid differences between the R3A and R3B Envs (Fig 3a). The crystal structure for the thermodynamically stable HIV-1 viral fusion protein contains 36 amino

acids of the HR-1 and 34 amino acids of the HR-2 (25). Only three of the R3A/R3B differences exist within the known structure. As reported that C34 helices pack outside the N36 helices in an anti-parallel fashion, our mapping suggests that the changes that map to the N and C-terminal Heptad Repeats are unlikely to contribute to direct HR1-HR2 interaction that could lead to an altered fusion phenotype. However, one upstream difference in the HR-1 (Q543R), as well as one downstream difference in HR-2 (E665K), could not be mapped on the known structure. Further, the difference that maps to the cysteine mini-loop region between the HRs also cannot be mapped (H620D), as this region does not exist within the crystal structure. Despite the mentioned limitations presented by the model, amino acid changes were inferred to exist on the outer regions of the helices, suggesting that an interaction due to mutation was not likely. Rather, individual changes may have conferred the observed fusion ability.

Site-directed mutagenesis of each amino acid site in R3A HR1-HR2 region was performed to determine if substituting individual R3B changes in the R3A background would impact the Env's ability to fuse (Fig. 3b). A cell-cell fusion assay was utilized to determine whether single changes in R3A extracellular domain would affect the high fusogenic phenotype. Retroviral vectors that express the various HIV-1 Envs and Tat were used to transduce BC7 cells. Upon co-culture with HOS/LTR-luciferase cells expressing the necessary receptors CD4 and CXCR4, fusion from Env-Cell fusion was quantified by luciferase activity due to Tat activation of LTR-luciferase. Our results show that R3A Env promoted levels of fusion on average 5 fold higher than R3B Env, and Envs with single point mutations had similar levels of fusion to the parental R3A Env. We concluded that

individual amino acid changes in the ectodomain did not affect the fusion ability of R3A Env (Fig 4a).

We have previously reported that both R3A and R3B Envs were equivalently sensitive to fusion inhibitor T20 (26) or C34 (data not shown). As T20 or C34 is derived from the HR-2 sequence and binds to HIV-1 Env HR-1 to inhibit fusion, we thus first focused on whether changes in the R3A HR-2 domain could alter fusion ability. To test the hypothesis that the R3A HR-2 domain contributes to enhanced fusion activity, chimeras were produced exchanging the HR-2 domains between R3A and R3B (Fig 3b). Interestingly, the R3A chimera containing the R3B HR-2 significantly reduced fusion ability while the R3B chimera containing the R3A HR-2 demonstrated a higher fusion function (Fig 4b).

To determine whether the exchange of HR-2 domains would affect virion-mediated fusion or viral entry into host cells, pseudotyped viruses with HIV-1 Envs and NL4-luc were generated and used to transduce SupT1 cells (Fig 4c). The data shows the increased entry of R3A is significantly attributed to the HR-2 domain, as viruses carrying the R3A HR-2 domain demonstrate enhanced ability to enter host cells, while viruses expressing R3B HR-2 demonstrate low entry ability.

It is possible that changes in the HR-2 provide an enhanced rate of fusion for the R3A Env, and hence more efficient entry. To determine this possibility, a time course to measure the rate of fusion of HIV-1 Envs was performed. BC7 cells expressing HIV-1 Envs were first assessed for equal HIV-1 Env surface expression by FACS analysis with the 2g12 anti-Env mAb (Fig 5a). Western blot analysis was also performed to show that all HIV-1 recombinant Env were similarly expressed and processed in transfected cells (data not shown). Equivalent numbers of Env/Tat-expressing cells were co-cultured with HOS/LTR-

luc reporter cells on ice, and then placed at 37°C. Fusion inhibitor C34 was added at 0, 6 and 12 hr to inhibit fusion. After 48hrs, cells were lysed and luciferase was measured to assess fusion. BC7 cells expressing R3A Env demonstrated an enhanced fusion activity constitutively over time, compared to R3B-expressing cell, and this enhanced fusion ability was correlated with the HR-2 domain of R3A (Fig 5b and data not shown). However, relative inhibition of fusion of Envs encoding the R3A HR-2 was more dependent on the early addition of C34 than the R3B Env. Thus, C34 added at 6 hr post initiation of fusion only inhibited 70% of fusion mediated by R3A or R3B/A-HR2, but still completely inhibited R3B-mediated fusion. When added at 12 hr post fusion initiation, C34 inhibited 40-50% of fusion by R3A or R3B/A-HR2 but still inhibited 80% of R3B-mediated fusion. The data suggests that the R3A Env, through its unique HR-2 domain, fuses with target cells more rapidly.

Our previous studies suggest that the enhanced CXCR4 binding affinity could be a determinant for pathogenicity of R3A (26). As the R3A HR-2 was determined to be the major contributor for fusogenicity, the domain's role in CXCR4 binding affinity was analyzed. Analysis of CXCR4 binding affinity was performed, and R3B/A Ecto recombinant showed intermediate resistance to AMD3100, compared to R3A and R3B (Fig 6). Further, the R3A/B-HR2 and R3B/A-HR2 showed similar CXCR4 affinity as their parent viruses R3A and R3B, respectively. As it has been shown that swapping of the HR-2 domain also exchanges fusion activity of ENV, there is no clear correlation between CXCR4 binding affinity and enhanced fusogenicity.

The HR-2 domain of the R3A gp41 plays a significant role in the enhanced pathogenic ability of R3A in the HFTOC model.

To address whether the R3A HR-2 also contributes to elevated CD4+ cell depletion in lymphoid organs, full-length HIV-1 viruses were generated that expressed chimera HR-2 Envs to compare against NL4-R3A and NL4-R3B. As previously reported with R3A and R3B in activated PBMC (26), similar replication and CD4 depletion activity was observed with all HIV-1 recombinants in activated PBMC (data not shown). HF-TOC was infected with parent and chimera viruses, and both viral replication and CD4+ cell depletion were assessed. Enhanced viral replication was seen by NL4-R3A over NL4-R3B, as observed previously (26). Interestingly, both R3A/B HR2 and R3B/A HR2 chimeric viruses showed higher levels of replication than R3B (Fig. 7a). Consistent with the fusion activity, chimera viruses with the R3A HR-2 domain were found to deplete CD4 thymocytes more efficiently compared to viruses with R3B HR-2 domain (Fig 7b). By Day 11 post infection, NL4-R3A was found to deplete CD4+ cells by 90%, while NL4-R3B or NL4-R3A/B HR-2 depleted cells only by ~30-40%. Interestingly, NL4-R3B/A HR-2 showed an intermediate level of CD4+ cell depletion, significantly more pathogenic than NL4-R3B but less pathogenic than NL4-R3A. These findings suggest that the R3A HR-2 contributes to the enhanced CD4+ cell depletion by R3A in the thymus organ, but additional determinants in the R3A ectodomain also play a role in enhanced pathogenesis. The entire ectodomain may function in concert to enhance HIV infection and pathogenesis seen by NL4-R3A in the HFTOC model. In addition, the fusion activity, but not the CXCR4 affinity, is correlated with the enhanced pathogenicity of R3A in lymphoid organs.

Discussion:

The virologic mechanisms by which human lymphoid organs undergo CD4+ cells depletion during HIV-1 infection remain incompletely characterized. We have previously reported that HIV-1-induced fusion is the major contributor to pathogenesis of both infected and uninfected cells in human lymphoid organs (26-28). In this report, we have investigated the virologic determinants that contribute to the elevated fusogenicity and pathogenicity of the R3A Envelope. We discovered that the gp41ectodomain of R3A Env determines its high pathogenic and fusion activity. As this domain carries 6 amino acid differences between the R3A and R3B Env, we further determined that the three mutations in the HR-2 domain of R3A Env play a major role in its elevated fusion activity, and partially contribute to the high pathogenic activity in the thymus organ model.

By comparing Env chimeras of a highly pathogenic virus with a less pathogenic virus, six amino acid differences within the ectodomain were mapped to confer the enhanced fusion and pathogenic ability of R3A. Individual site-directed mutants did not impact the enhanced fusion phenotype of R3A Env (Fig. 4). Thus, a functional determinant consisting of redundant or complementary residues in R3A ectodomain or HR-2 is implicated in contributing to the enhanced fusogenic activity.

R3A and R3B Envs were similarly sensitive to fusion inhibition by T20 or C34 (26). As T20 or C34 is an HR-2 homolog which functions to bind to HR-1 and inhibit fusion, the changes in the R3A HR-1 region were probably not involved in the elevated fusion activity. Consistent with the prediction, the three amino acid differences in the HR-2 domain of R3A were necessary and sufficient to confer the enhanced fusion ability of R3A Env. Three functional domains have been reported within the HR-2 of the gp41 ectodomain: an HR-1

binding domain (628-666), a pocket binding domain (628-635), and lipid binding domain (666-673)(19). Interestingly, all three differences in the HR-2 between R3A and R3B Envs are encoded within the HR-1 binding domain. The HR-1 binding domain of R3A Env is thus a major determinant of enhanced fusion activity.

In addition to the elevated fusion activity associated with the R3A Env, HIV-1 recombinants containing the R3A ectodomain depleted thymocytes equivalently to parental NL4-R3A, suggesting that enhanced fusion may contribute significantly to pathogenesis. Because increased CXCR4 binding efficiency was previously suggested also as a determinant for thymic pathogenicity (26), the R3B/A Ecto Env was assayed for relative CXCR4 binding efficiency (Fig 6). As was seen with other chimera Envs (expressed in pseudotyped virus)(26), an intermediate level of CXCR4 binding efficiency was observed. This intermediate phenotype was shared with chimera Envs that demonstrated significantly less thymic pathogenicity, suggesting that co-receptor binding efficiency and sensitivity to co-receptor antagonist AMD-3100 do not directly correlate to the observed pathogenesis of the Env.

It is thought that the HR-2 domain functions to "zip" over the HR-1 domain after the initial step of Env-cell fusion, so the amino acid changes seen in the R3A HR-2 may allow a more rapid ability to fuse the envelope to the cell, providing for significantly enhanced ability for the Env to enter the cell as well. It is possible that changes present in NL4-R3A provide for an enhanced rate of fusion of the Env, which allows for enhanced pathogenesis compared to NL4-R3B. To provide various "snap shots" of fusion over time, fusion inhibitor C34 was used to inhibit fusion at various time points. Envs expressing R3A HR-2 showed enhanced and accelerated fusion compared to Envs expressing R3B HR-2. Envs expressing

the R3A HR-2 provided more robust fusion ability, with a significant difference in slope of kinetics between R3A and R3B Envs.

We demonstrate that the HR-2 is an important contributor for both fusion and pathogenic activity in a relevant human lymphoid organ model. All three of the mutations in R3A HR-2 region lead to changes in hydrophobicity and charge. Specifically, the R3A HR-2 carries changes to serine, asparagine and glutamic acid, which are all hydrophilic. The hydrophilic nature of this region may shed light on the highly fusogenic ability of the R3A HR-2. Also, comparing R3A to R3B, the H to D at position 620 removes a partially positive charge and replaces it with a negative charge, and E to K at position 665 removes a negative charge and replaces it with a positive charge. The HR-2 differences provide various changes that may affect the conformation of the six helix bundle, such that R3A HR-2 may interact differently with the viral membrane than R3B HR-2. We analyzed HIV-1 sequences in the Los Alamos National Laboratory HIV Sequence Database for their sequence comparison with R3A and R3B. Of approximately 500 Clade B Envs, the Asp-651 in R3A Env is the consensus whereas the Ile-651 in R3B is not reported in other subtype B HIV-1 sequences. Interestingly, the Lys-665 in R3B is the consensus but Glu-665 of R3A is not detected in the other subtype B HIV-1 strains. The amino acid 665 is the first position of the Membrane-Proximal External Region (MPER), which is known to be a relatively conserved region that plays a distinct role in fusion and is a target for anti-viral drugs (35). It is possible that this R3A mutation, in combination with other mutations in the HR-2 region, may contribute to an enhanced fusogenic phenotype.

Several groups have reported that HIV-1 Env mediated fusion is the primary determining factor in CD4 T cell loss (4, 11, 12). An enhancement in the ability of the Env

to fuse would lead to an increase in the efficiency of new infections, at the cost of higher amounts of cells death. Indeed, we have reported the enhanced pathogenicity of a highly fusogenic virus in the fetal thymus organ model. Further, we demonstrate that enhanced fusion by R3A is directly due to the HR-2 domain. The data from these experiments suggest that the entire structural change is necessary for R3A's HR-2 mediated effect. Interestingly, pathogenesis conferred by the R3A ectodomain is not strictly due to the changes seen in the HR-2, so other changes observed in the ectodomain including HR-1 may contribute to the overall pathogenesis seen by R3A. Future experiments will be performed to determine interactions that may exist between the HR-1 and HR-2 domains that may provide this enhanced fusion-mediated pathogenesis observed with the R3A ectodomain.

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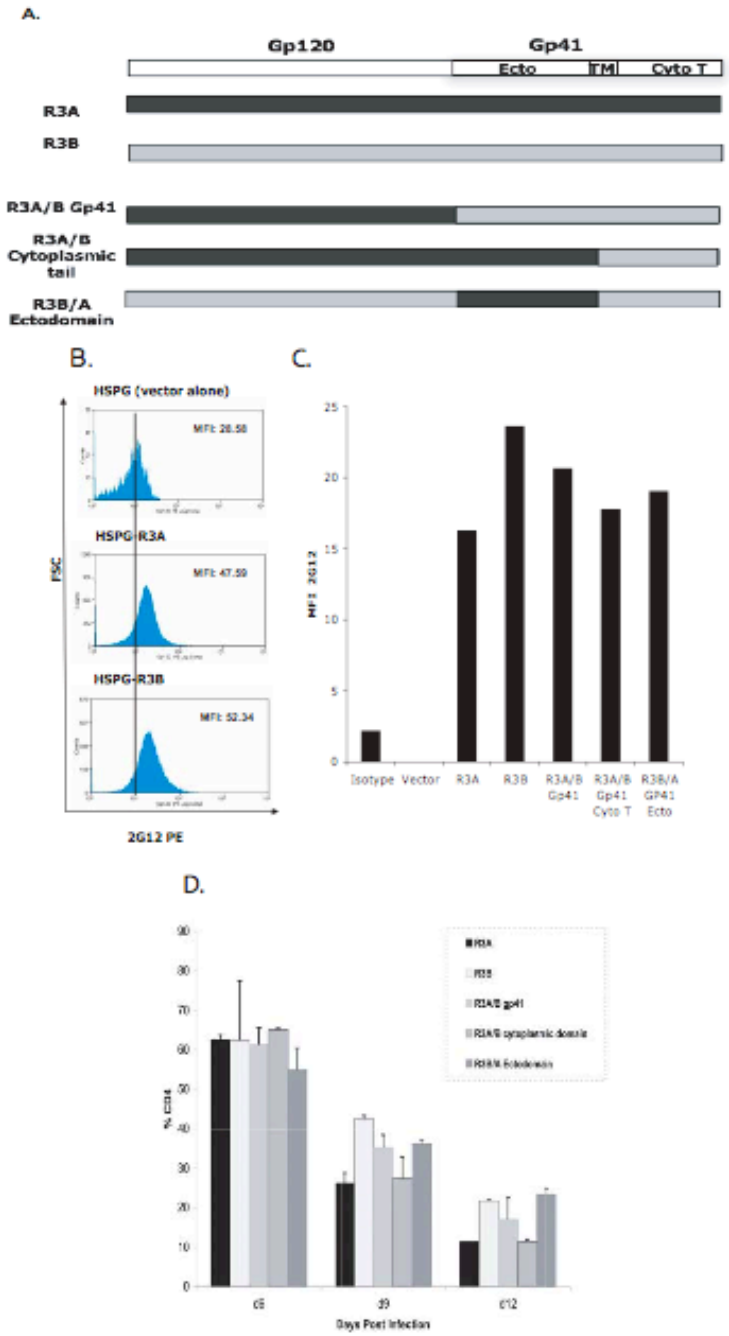
A.
Gp120
Gp41
Ecto
TM
Cyto T
R3A
R3B
R3A/B Gp41
R3A/B Cytoplasmic tail
R3B/A Ectodomain
B.
HSPG (vector alone)
MFI: 28.58
HSPG-R3A
MFI: 47.59
HSPG-R3B
MFI: 52.34
FSC
2G12 PE
C.
MFI 2G12
Isotype
Vector
R3A
R3B
R3A/B Gp41
R3A/B Gp41 Cyto T
R3B/A GP41 Ecto
D.
% CD4
Days Post Infection

Figure 1.

Figure 1: R3A, R3B and chimera recombinants. (A) A schematic diagram of R3A and R3B Env, and their gp41 chimeric recombinants. Six amino acid differences exist between R3A and R3B within the the Ectodomain, and four within the Cytoplasmic tail domain. (B) Cell surface expression of Env proteins is equivalent between R3A, R3B and the chimeric Env. 2G12 mAb staining was performed for FACS analysis of 293T cells transfected with Env-expressing vectors. Relative expression levels are presented as Mean Fluorescence Intensity (MFI). (C) R3A, R3B and their HIV-1 recombinants show similar infection and CD4 T cell depletion in activated PBMC. Activated PBMCs were infected with HIV-1 stocks with parental or chimera R3 Env genes. Relative CD4 T cell depletion (with mock samples as 100%) is shown at 6, 9 and 12 days post infection. Error bars represent standard deviations from triplicate samples.

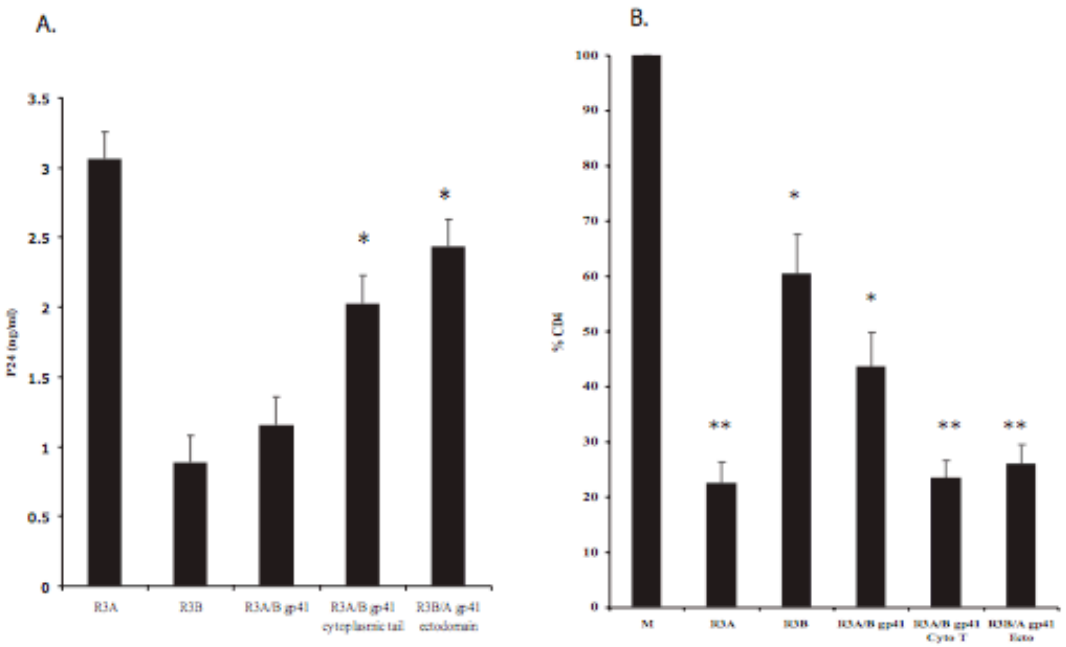
A.
P24 (ng/ml)
3.5
3
2.5
2
1.5
1
0.5
0
R3A
R3B
R3A/B gp41
R3A/B gp41 cytoplasmic tail
R3B/A gp41 ectodomain
*
*
B.
% CM
100
90
80
70
60
50
40
30
20
10
0
M
R3A
R3B
R3A/B gp41
R3A/B gp41 Cyto T
R3B/A gp41 Ecto
**
*
*
**
**

Figure 2.

Figure 2: The R3A ectodomain determines its higher pathogenic activity in vivo. HIV-1 viruses encoding the R3A, R3B or the chimeric Env genes were used to infect HF-TOC. (A) HIV-1 replication was measured from supernatants harvested from HF-TOC at 7, 9 and 11 dpi. Shown are combined data at 7-9 dpi. (B) Relative HIV-1 pathogenesis (depletion of total CD4+ thymocytes) in HFTOC was assessed by FACS analysis, with mock (M) sample at 100%. Data demonstrate combined results from six independent experiments. Error bars represent standard deviations. $*$, $p<0.05$ relative to mock; ** $p<0.01$, relative to R3B.

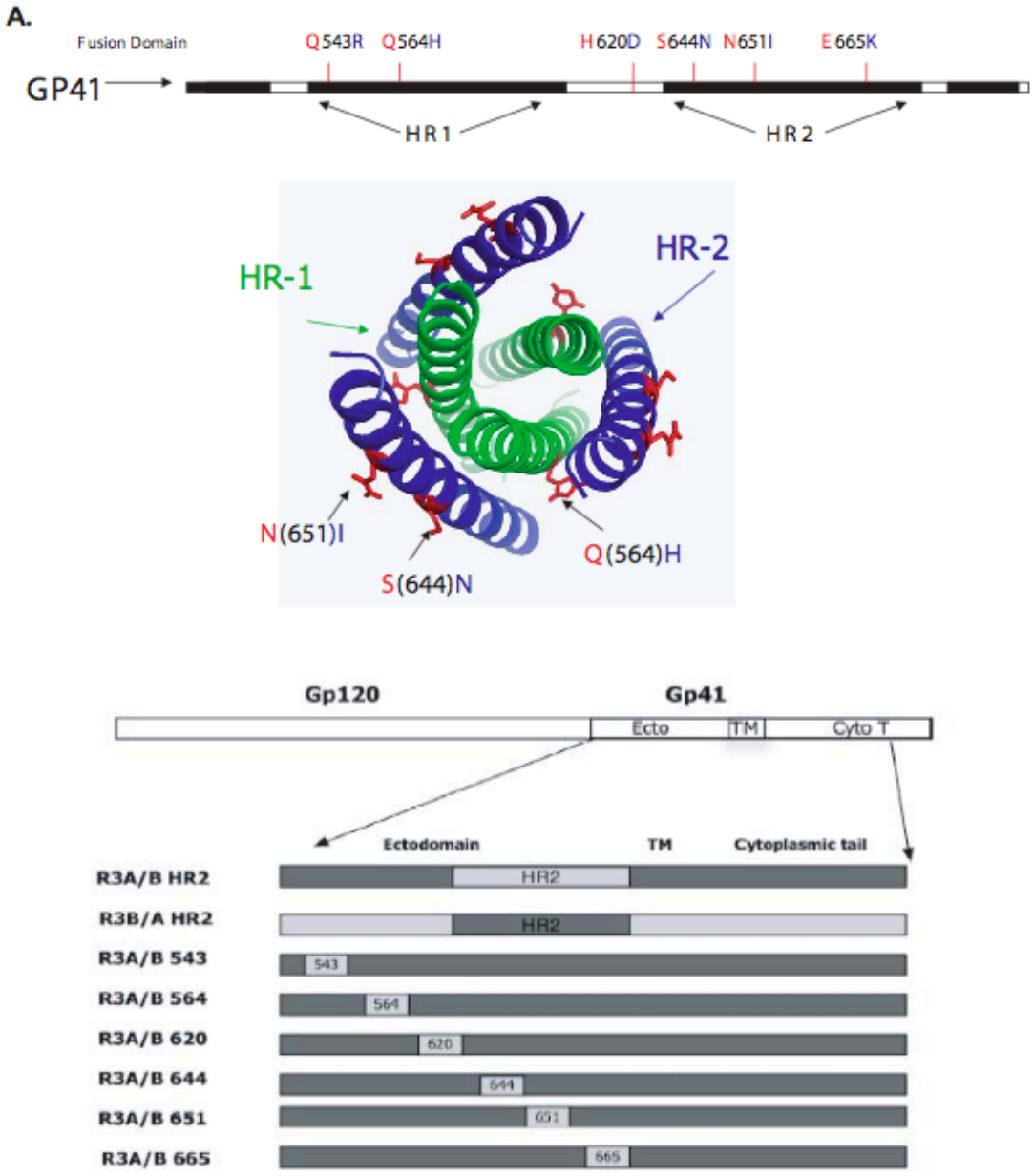
A.
Fusion Domain
Q543R
Q564H
H620D
S644N
N651I
E665K
GP41
HR1
HR2
HR-1
HR-2
N(651)I
Q(564)H
S(644)N
Gp120
Gp41
Ecto
TM
Cyto T
Ectodomain
TM
Cytoplasmic tail
R3A/B HR2
R3B/A HR2
R3A/B 543
R3A/B 564
R3A/B 620
R3A/B 644
R3A/B 651
R3A/B 665
HR2
HR2
543
564
620
644
651
665

Figure 3.

Figure 3: Ectodomain recombinants of R3A and R3B. (A) A Ribbon Diagram depicts the changes seen between R3A and R3B in the ectodomain. Only 3 of the 6 differences between R3A and R3B can be modeled upon the known crystal structure. These changes do not seem to change direct interactions between HR-1 and HR-2 domains. (B) The HR-2 domains were swapped between R3A and R3B Env genes. Individual amino acids in the R3A Env gene were also changed to R3B residues using site directed mutagenesis.

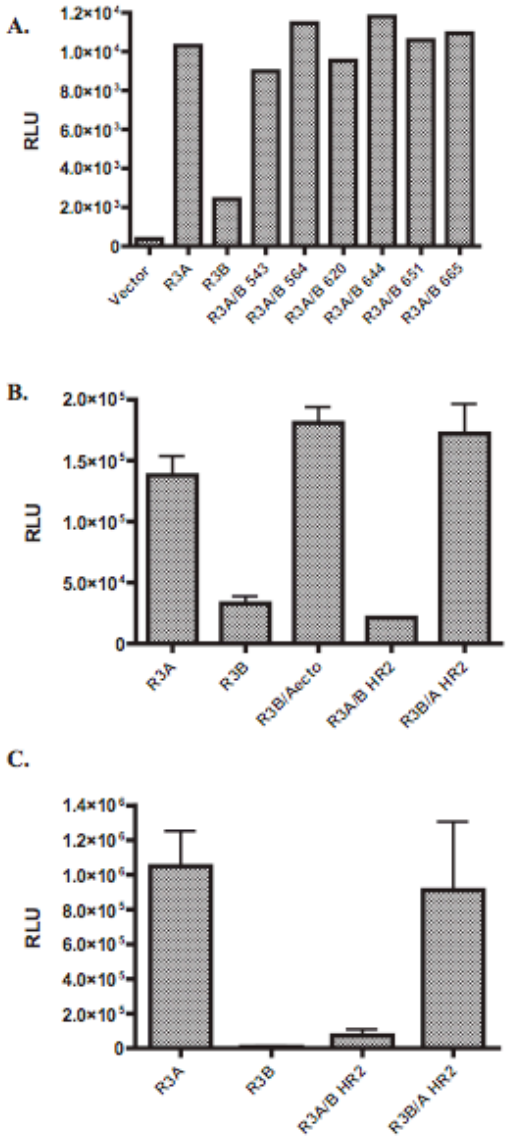
A.
RLU
1.2×10⁴
1.0×10⁴
8.0×10³
6.0×10³
4.0×10³
2.0×10³
0
Vector
R3A
R3B
R3A/B 543
R3A/B 564
R3A/B 620
R3A/B 644
R3A/B 651
R3A/B 665
B.
RLU
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
R3A
R3B
R3B/Aecto
R3A/B HR2
R3B/A HR2
C.
RLU
1.4×10⁶
1.2×10⁶
1.0×10⁶
8.0×10⁵
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
R3A
R3B
R3A/B HR2
R3B/A HR2

Figure 4.

Figure 4: The HR-2 domain of R3A determines its enhanced fusion activity. BC7 cells expressing various HIV-1 Tat and Env genes were co-cultured with cells expressing CD4, CXCR4, and LTR-luciferase. Relative fusion is measured by the activation of HIV-LTR-luciferase (RLU) by Tat in the fused cells. (A) Individual amino acid changes in R3A Env gene do not significantly affect the fusion phenotype of R3A. Site directed mutations of R3A ectodomain carrying R3B changes were assayed, and individual changes were not seen to confer an R3B-like phenotype. (B) Swapping of the HR-2 domain completely exchanged the fusion phenotype in the cell-cell fusion assay. (C) Swapping of the HR2 domain also exchanges the HIV-1 virus-cell fusion. NL4-luc pseudotyped with various R3 Env proteins was used to infect SupT1 cells. Luciferase expression (RLU) was measured at 48 hr. post infection. Error bars represent samples in triplicates.

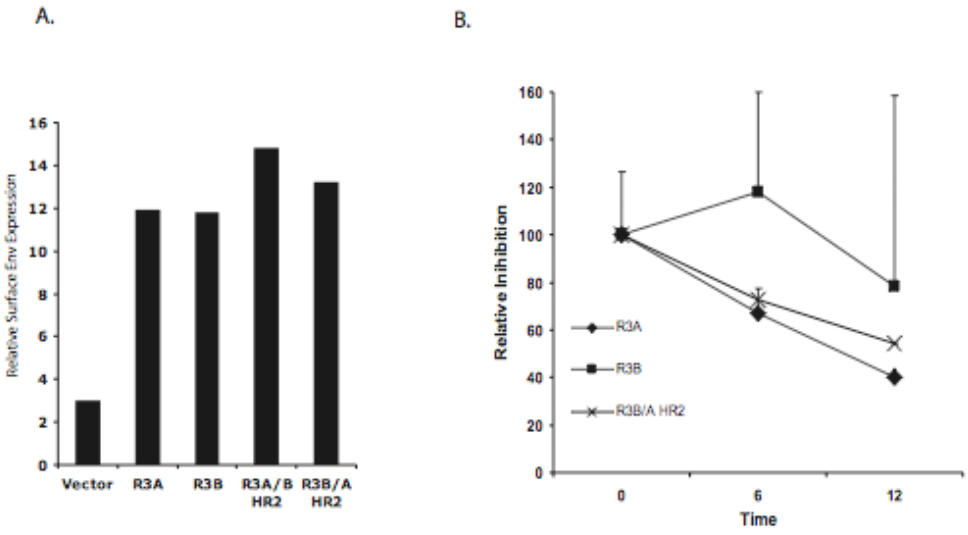
A.
B.
Relative Surface Env Expression
16
14
12
10
8
6
4
2
0
Vector
R3A
R3B
R3A/B HR2
R3B/A HR2
Relative Inhibition
160
140
120
100
80
60
40
20
0
R3A
R3B
R3B/A HR2
0
6
12
Time

Figure 5.

Figure 5: The R3A HR-2 domain confers accelerated fusion activity. (A) Equivalent expression of R3A, R3B and their HR-2 chimeric recombinant Env proteins on the surface of transduced BC7 cells. Surface expression of HIV Env on transduced BC7 cells was assessed by 2G12-FACS analysis. Relative expression levels are presented as Mean Fluorescence Intensity (MFI). (B) Recombinant Env genes encoding the R3A HR-2 demonstrated accelerated fusion. Cells expressing various HIV Env genes were mixed with the fusion reporter cell line. Fusion inhibitor C34 was added at the indicated time after co-culture at 37^{o}C. Inhibition of fusion of each recombinant Env by C34 when added at time point 0 is calculated as 100% inhibition. Relative inhibition by C34 added at 6 and 12 hr is shown. Data is representative of two independent experiments and error bars represent standard deviations derived from duplicate samples.

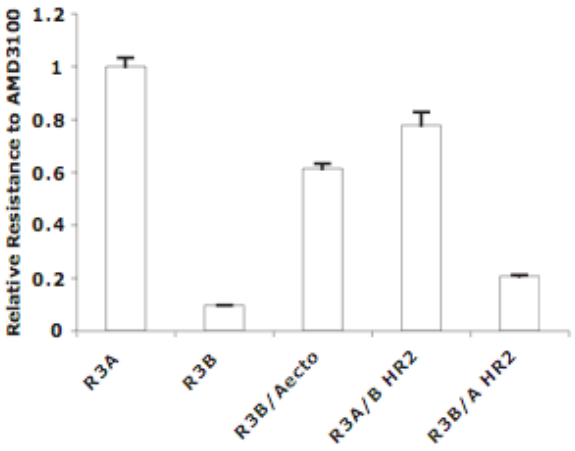
Relative Resistance to AMD3100
1.2
1
0.8
0.6
0.4
0.2
0
R3A
R3B
R3B/Aecto
R3A/B HR2
R3B/A HR2

Figure 6.

Figure 6: The enhanced CXCR4 binding affinity of R3A Env is not correlated with the enhanced fusion activity. Resistance of each HIV-1 Env gene to AMD3100 (200 nM) relative to R3A was determined. Error bars represent standard deviations derived from triplicate samples.

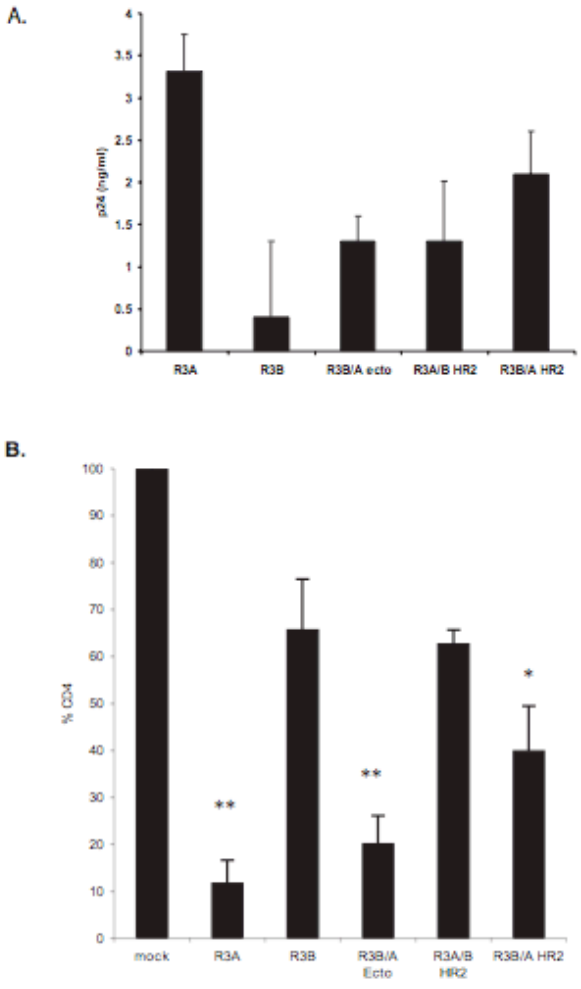
A.
p24 (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
R3A
R3B
R3B/A ecto
R3A/B HR2
R3B/A HR2
B.
% CD4
100
90
80
70
60
50
40
30
20
10
0
**
**
*
mock
R3A
R3B
R3B/A Ecto
R3A/B HR2
R3B/A HR2

Figure 7.

Figure 7: The R3A HR-2 domain contributes significantly to its enhanced pathogenesis in human lymphoid organs. R3A, R3B and the chimeric recombinants were used to infect HF-TOC. (A) Viral replication was measured by p24 ELISA at 7, 9 and 11 days post infection. Shown are data from 7-9 dpi. (B) CD4+ thymocyte depletion was measured by FACS analysis at 11 days post infection of HFTOC with NL4-R3A, R3B and chimeras. Error bars represent standard deviations from samples in triplicate, and two independent experiments were performed with similar results. *, $p<0.05$ in comparison to R3B; **, $p<0.001$ vs. R3B.

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Chapter 3

Activation of pDC by A Pathogenic HIV-1 Env Contributes to T Cell Depletion in Human Lymphoid Organs: role of elevated CD4 binding affinity

(Publication in Preparation for Submission)

Abstract:

Activation of pDC and Type 1 Interferons (IFNs) play an important role in innate immune responses against viruses, by inhibiting viral replication while at the same time signaling the host adaptive immunity to respond to infection. The role of pDC and IFNa in HIV-1 infection and pathogenesis is not clear. A pathogenic HIV-1 isolate from a rapid progressor was found to induce high levels of type 1 IFN early in infection, and another genetically similar (but less pathogenic) viral isolate showed no significant induction of IFN. Depletion of pDCs from PBMC abolished the IFN induction, and purified pDCs were also preferentially activated by the pathogenic HIV-1 isolate. Interaction of the Env with CD4, as well as endocytosis was required for the activation. On the other hand, HIV-1 Env-mediated fusion and reverse transcription were not critical for the IFN induction. We find that the enhanced CD4 binding efficiency of the pathogenic HIV Env (correlated to the V1V2 region of HIV-1 ENV) plays an important role in HIV-induced Type 1 IFN, both *in vitro* and *ex vivo*. Finally, we find that Type 1 IFN contributes significantly to the depletion of human T cells in human lymphoid organs in a fusion independent fashion. We conclude that activation of pDC and its induction of IFNa play a significant role in HIV-1 induced T cell depletion.

Introduction:

HIV-1 causes significant impairment and depletion of CD4 T cells, ultimately progressing to immunodeficiency and AIDS. Multiple hypotheses exist to explain the gradual depletion of CD4+ lymphocytes during the chronic phase of infection. While the mechanisms for HIV-1 infection are well understood, the mechanisms for T cell loss and CD4 T cell death remain unclear. The frequency of infected cells that circulate though the peripheral lymphoid tissue as well as viral load do not account for the loss of CD4+ lymphocytes observed, so direct infection is discounted as a major contributor to depletion. HIV-1 infection results in T lymphocyte activation, which exists throughout the entire course of infection. This chronic immune activation is suggested as a major factor in the depletion observed. Differences in the pathogenic phenotype of SIV infection between the Rhesus Macaque and Sooty Mangabee corroborate with the idea that chronic immune activation may be an important requirement for immune depletion. Sooty Mangabees serve as the natural primate host of SIV, and can carry SIV at high viral loads with little to no discernable HIV-like immune activation or CD4 T depletion(43). Rather, infection of Rhesus Macaque with SIV develops both significant immune activation and pathogenesis, ultimately leading to AIDS-like disease.

Recently, our group and others have reported mechanisms where HIV-1 undergoes fusion to induce apoptosis in infected and uninfected cells (22, 36). It is possible that HIV-1 may induce other mechanisms of non-fusion mediated uninfected cell death of T lymphocytes. In fact, the dominant proportion of CD4+ and CD8+ lymphocytes in the peripheral blood and lymph nodes of infected patients are observed to be both uninfected and

apoptotic, suggesting that other methods may be utilized by HIV-1 to deplete cells in the organs and the periphery(22).

HIV-1 infection of T lymphocytes first requires the binding of the receptor CD4 to orchestrate the binding of a co-receptor (either CCR5 or CXCR4), ultimately leading to virus-cell fusion. Recent studies indicate that HIV-1 also uses the receptor CD4 to bind to plasmacytoid dendritic cells (pDCs), although endocytosis (rather than fusion) is suggested as the primary method of entry for virions into pDCs(4, 32). Entry into the pDC endosome by the HIV-1 virion induces the secretion of significant amounts of Type 1 IFN from the cells, through activation of Toll-like Receptor 7(4, 30). This IFN induction provides a robust anti-viral response to many pathogens, which in turn activates the IFN signaling pathway through binding of IFN α/β receptors that are present on most cell types. Along with amplification of Type 1 IFN, an arsenal of anti-viral IFN-associated proteins is produced due to this signaling pathway, which typically provides an effective anti-viral environment to induce immunological control of infection. Indeed, the fact that most viruses employ methods to antagonize either IFN induction of amplification clearly indicates the important anti-viral functions that Type 1 IFNs play to control viral replication and spread.

Activation of pDC is reported in a number of viral infections, including influenza, SARS, HBV and HCV. It is believed that pDC play a critical role in controlling viral infection and/or in clearing the virus, although the precise mechanism is not clear. Several lines of evidence have also indicated that pDC cells may be important in HIV-1 infection and pathogenesis (5, 9, 13). First, pDC express high levels of CD4, CCR5 and CXC4 and HIV-1 can productively infect pDC cells *in vitro*. Second, pDC (but not mDC) are efficiently activated by HIV-1 in the absence of a productive infection(13). Third, HIV+ patients are

usually associated with lower levels of pDC activity. In fact, early study and discovery of human pDC were focused on the finding that IFN-producing cells are reduced in AIDS patients (42). Also, non-pathogenic SIV infection in the Sooty Mangabey is associated with stable levels of pDC whereas reduced pDC levels are reported in SIV-infected Rhesus Macaques during late chronic stages of infection (11, 43). A decrease in pDC number and activity occurred, correlated with elevated plasma viremia and immune activation(29). The pDC, therefore, are likely critical modulators of HIV infection. Mixed results from both *in vitro* and *in vivo* studies further complicate the role of pDCs and IFNs in HIV-1 infection. *In vitro* studies have demonstrated that Type 1 IFNs inihbit HIV-1 viral replication, although several *in vivo* studies and clinical trials have engendered mixed results in efficacy of IFNα treatment and control of HIV-1(28, 38-40). This has led the question of whether to use IFNα as a therapeutic for HIV-1 infection to remain unresolved to this point. This is further complicated by recent work that suggests that IFNα produced by pDC is implicated as a mediator in bystander cell death observed *in vitro* as well as in clinical analysis(18-21).

The study of pDCs and their role in HIV-1 pathogenesis is greatly limited the absence of an adequate experimental model for HIV infection. As pDCs represent a minor (<1%) cell population in the periphery, they pose a difficulty for experimentation. Activation of PBMCs *in vitro* for infection will enhance depletion of non-lymphoid cells, while at the same time placing the cells in an artificially activated state that doesn't necessarily represent the *in vivo* setting of the lymphoid microenvironment. Several groups have utilized lymphoid tissue such as tonsil, lymph node and thymus as a more adequate *ex vivo* model for acute infection pathogenesis, in the absence of an adaptive immune response(6, 7, 18, 25, 27). Specifically, we and others have shown that HIV infection and depletion of thymic tissue closely models

in vivo infection in terms of viral replication and CD4+cell depletion while at the same time being refractory to laboratory adapted HIV strains(12, 31, 35, 37). It has also been reported that pDCs are present in thymic tissue, and play a relevant role in IFN induction and IFN-Stimulated Gene (ISG) expression(15, 26).

Recently, viruses isolated from a rapid progressor patient in the ALIVE cohort have been studied for their role in the rapid progression to AIDS seen in the patient. The isolate (denoted as R3A) was found to be significantly pathogenic in the human fetal thymus organ culture (HFTOC) model, a relevant model for HIV-1 pathogenesis(34). We report here that the pathogenic HIV-1 isolate R3A can efficiently activate pDC and induce Type 1 IFN expression in resting PBMC and in human thymus organ culture (HFTOC) model. The activation requires Env interaction with CD4, but does not require productive HIV-1 infection or even fusion. The V1V2 region of R3A Env that determines the high CD4 binding affinity also determines its pDC activation in PBMC and in HFTOC. In addition, the induction of IFNα/β contributes to the elevated T cell depletion activity of R3A that is fusion-independent. Therefore, we have identified a new pathogenic determinant of HIV-1 Env that is involved in activating pDC and inducing Type 1 IFNs. Blocking Env-mediated fusion and IFN efficiently prevent HIV-1 mediated T cell depletion in the human thymus organ.

MATERIALS AND METHODS:

Cells and Reagents: A293T and Magi-CXCR4 cells (was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: U373-MAGI-CXCR4CEM from Dr. Michael Emerman)(44), and A549 cells (obtained from the Carolina Vaccine Institute) were cultured in Dulbecco's Modified Eagle medium supplemented with 10% (vol/vol) bovine calf serum (Sigma Chemical, St. Louis, Mo.) and 100 μg of penicillin and streptomycin/ml. All viral isolates were prepared and stored in Iscove's Modified Dulbeccos Media supplemented with 10% (vol/vol) bovine calf serum and 100 μg penicillin and streptomycin/ml. Peripheral blood monocytes were purified from the blood of healthy HIV-1 negative donors by Ficoll-Plaque density gradient centrifugation and cultured in Iscoves Modified Dulbeccos Media supplemented with 10% bovine calf serum and 100 μg penicillin and streptomycin/ml. Peptides C34 were synthesized by at the MicroChemistry Laboratory of the New York Blood Center (Kindly provided by Dr. S. Jiang). C34 was reconstituted in phosphate buffered saline at a concentration of 1 mg/ml. Anti-HIV drug Nevirapine (as obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) and antibody B12 (was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody (IgG1 B12) from Dr. Dennis Burton and Carlos Barbas)(3, 8) as well as monoclonal antibodies raised against human IFNs α and β were obtained through the NIH AIDS Research and Reference Reagent Program. Chloroquine and Bafilomycin A1 were obtained commercially (Sigma).

Plasmids and Virus Preparation. Isolation of ENV from primary isolate and cloning of NL-R3A and NL-R3B have been previously described(33, 34). Infectious virus for R3A, and

R3B chimeras were derived as follows: chimeric Envs were cloned into p83.10 plasmid containing partial NL4 genome (EcoRI to XhoI) (was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p83-10 from Dr. Ronald Desrosiers)(14). Initial cloning inserted a missence mutation into the NL4.3 Nef gene, but was repaired by PCR strategies. Chimera Envs were developed using the restriction sites DraIII and AccI, which were used to exchange the V1, V2, and C2 domains of Envs, between R3A and R3B in the p83.10 vector. Full-length infectious viral DNA was constructed by then cloning the EcoRI and XhoI fragments into the NL4-3 backbone. Plasmids were transfected into A293T, and then supernatant was harvested at 48 hrs post transfection, and co-cultured with PHA/IL-2 stimulated PBMCs.

Viral Quantitation. Gag was detected in viral stocks as well as supernatant from HFTOC infections using a p24 enzyme-linked immunosorbent assay (ELISA) kit (AIDS Vaccine Program, NIH). Viruses were quantified for infectious units/ml by infection of Magi (CXCR4) cells, as previously reported.

Cell Sorting. Plasmacytoid Dendritic cells were isolated from PBMCs using CD303 (BDCA4) isolation kit and an AutoMACS magnetic system (Miltenyi Biotech).

Interferon Analysis. Resting PBMCs were infected with NL-R3A and NL-R3B (and chimeras) at 10-100 ng/ml p24 for 12-16 hours. Supernatants were harvested, acidified (to removed infectious HIV-1 and analyzed for Type 1 IFN expression using an IFN Bioassay and IFNα ELISA (Bender Systems). The IFN Bioassay was performed as follows: Supernatants harvested from infections were over-layed on A549 cells. 24 hours later, interferon-sensitive EMCV was added to A549 cells. After 18hrs, remaining viable cells (due to protection by IFNα/β in the supernatant) were quantified in comparison to known IFN

Standard (NIH AIDS Research and Reference Reagent Program). Viable cells were removed from plated by Trypsin Reagent (Sigma Chemical, St. Louis and quantified using Guava Easycyte Analysis (Guava Technologies).

Human Fetal Thymus Organ Culture. The procedure for infection and culture of human fetal thymus has been previously described (33-35). In brief, human fetal thymuses (19 – 22 weeks of gestation) were dissected into ~ $2mm^2$ fragments, using a dissecting microscope to retain the lobe structure for each fragment.

Four fragments were placed on organotypic culture membranes (Millipore) and under-laid with HFTOC media (previously described) were plated in a 6 well tissue culture plates. Thymic fragments were infected with equivalent amounts of virus (100-800 IU) in 15 ul per fragment. Viral and mock supernatants were derived from the same donor sample per experiment.

Thymic fragments were cultured at 37°C in 5% $C0_2$ for the length of each experiment. At harvest day of experiments, thymic fragments were disassociated in 350ul PBS with 2% FBS using pestels (Bellco Co.) for Florescent-Activated Cell Sorting (FACS) Analysis.

FACS Analysis. Thymocytes were harvested and stained for CD3+, CD4+, CD8+ and viability using Fluorescence labeled antibodies (Caltag and Invitrogen) and analyzed on a Cyan Fluorescence Cytometer, using Summit software.

Results:

The highly pathogenic NL-R3A preferential activates pDC *in vitro*.

We have previously demonstrated that NL-R3A is highly pathogenic in the human fetal thymus organ culture model (HFTOC) or SCID-hu Thy/Liv mice in vivo, characterized by massive CD4+ thymocytes depletion. Further, fusion-mediated apoptosis has been identified as a contributor to cell death observed in the HFTOC model (36). To further elucidate how the pathogenic NL-R3A contributes to elevated pathogenesis, we measured Type 1 IFN induction and pDC activation by the pathogenic NL-R3A and the less pathogenic NL-R3B recombinants (described in (36)) *in vitro*, using resting PBMC and a type 1 IFN bioassay that detects all Type 1 IFNs (**Fig. 1a**). Correlated with their pathogenic activity in lymphoid organs, NL-R3A, but not NL-R3B, induced robust production of IFN, assayed by protection of cell killing mediated by IFN-sensitive viruses as reported (10). The activity was shown to be type I IFN, as neutralizing antibodies for IFNα/β efficiently neutralized the activity (**Fig 1b**). We further confirm that human IFNα was induced by NL-R3A infection as measured by ELISA (**Fig 1c**). Interestingly, expression of IL-6 (measured by ELISA) was also preferentially induced by NL-R3A infection, suggesting that R3A ENV may preferentially activates pDC cells in PBMC (**Fig 1d**).

As pDCs are the major rapid IFN inducing cells in response to viral infections, we tested if pDC are the target cells that express IFN in response to NL-R3A infection. First, we depleted pDC (BDCA4+) cells with the MACS bead kit by >95% (**Fig 2a**). The remaining PBMC cells were challenged with NL-R3A or R3B as above. Interestingly, NL-R3A failed to induce any IFN from the pDC-depleted PBMC (**Fig 2b**), suggesting that pDC cells are the major target cells of R3A infection that leads to IFN induction. Furthermore, when purified

pDC (BDCA4+) cells were tested, NL-R3A also preferentially activated pDC relative to NL-R3B (Fig. 2c). Therefore, HIV-1 with the pathogenic R3A Env can efficiently activate pDC and induce IFN expression.

Activation of pDC by the highly pathogenic R3A requires CD4 interaction and endocytosis, but not fusion or productive HIV-1 infection.

The induction of IFN by NL-R3A was sensitive to B12 mAb, soluble CD4 and Leu3a mAb, which all block Env interaction with CD4 (**Fig. 3**). HIV Env-mediated fusion or productive infection of NL-R3A was not required for this activation, as a fusion inhibitor (C34) or an RT inhibitor (Nevirapine) showed no effect towards IFN induction by NL-R3A. Consistent with the idea that endocytosis is required for pDC activation and IFN induction, Chloroquine (that blocks early acidification of endosome) and Bafilomycin (late endocytosis/vacuolar ATPase) both completely suppressed the induction of IFN by NL-R3A. Thus the NL-R3A Env probably binds to pDC more efficiently (via CD4) and mediates endocytosis of HIV virions/genomes to activate TLR7 in the endosome of pDC.

Activation of pDC cells by R3A is determined by its V1V2 domains, correlated with elevated CD4 binding affinity.

Taking advantage of the recombinants between R3A and R3B (**Fig 4a**)(36), we defined the R3A Env determinants that are involved in pDC activation. Our results showed that the V1V2 region of the R3A Env determines this activity, correlating with the domains' function in enhanced CD4 binding affinity(33). R3A-R3B recombinants were used with resting human PBMC (**Fig. 4b**) or purified human pDC cells (**Fig. 4c**), and human type 1

IFN was measured by the bioassay. Thus the V1V2 region of R3A plays a major role in activating pDC cells because R3B with the R3A V1V2 region and R3A with the R3B V1V2 region efficiently swapped phenotypes in activating pDC. This is probably due to the elevated binding affinity for CD4, which determines pDC activation (17, 33).

Preferential activation of pDC and IFN induction by R3A in HF-TOC and is correlated with elevated levels of human T cell depletion in HIV-infected human thymus organs.

We also measured IFN induction by NL-R3A or R3B in the human fetal thymus organ culture (HF-TOC) model. Induction of IFN was readily detected in HFTOC media 24 hours after infection with R3A, but not with R3B (**Fig 5a**). The R3A V1V2 domain also contributes to IFN induction in human lymphoid organs. When tested in the human fetal thymus organ culture (HFTOC) model, the R3B/A-V1V2 recombinant demonstrated IFN induction activity in lymphoid organs similar to activation of pDCs (**Fig 5b**).
We defined the relative replication and pathogenicity of the R3B/A-v1v2 recombinant in comparison to R3A and R3B (**Fig. 5c**). Interestingly, R3B/A-V1V2 showed similar replication activity as R3A, significantly higher than R3B in HFTOC. When human thymocyte depletion was measured, R3B/A-V1V2 showed elevated pathogenic activity compared to R3B, but is significantly lower than R3A (**Fig. 5c**). Therefore, the V1V2 domain (and its pDC activation activity) of R3A is one of the R3A pathogenic determinants. Additional pathogenic determinants such as the elevated fusion activity are clearly encoded by R3A Env.

IFN induction by R3A contributes to elevated levels of human T cell depletion in HIV-infected human thymus organs.

We evaluated the role of type 1 IFNs in HIV-1 mediated pathogenesis in HFTOC. Fusion inhibitor C34 was also tested in combination with IFN nAb to study the role of IFN in depleting human CD4 T cells. Consistent with the anti-viral activity of IFNs, neutralization of IFNs significantly enhanced HIV-1 replication in HFTOC (**Fig 6a**). Interestingly, blocking IFN with neutralizing mAb (nAb) alone, although elevated HIV-1 replication, slightly prevented NL-R3A mediated T cell depletion (**Fig 6b**). Since the fusion activity of R3A Env is a major determent of human T cell depletion, the effect of blocking IFN in the presence of high levels of R3A Env-mediated fusion was not significantly detectable. However, when HIV-mediated fusion was also inhibited during peak viral replication, IFN nAb synergistically rescued human thymocytes (**Fig 6b**). We conclude that induction of IFN by NL-R3A infection contributes to its highly pathogenic activity, via a fusion-independent mechanism of T cell killing.

Discussion:

Various cellular interactions with HIV-1 contribute to the overall host pathogenesis. Here, we have shown that the pathogenic HIV-1 R3A Env can efficiently interact with CD4 to activate pDC in both resting PBMC and HFTOC. More importantly, HIV-1 mediated induction of IFN contributes to thymocyte depletion in HFTOC model, in a fusion-independent fashion. The V1V2 domain of R3A Env plays a critical role in this activity via enhanced CD4 binding. We conclude that the pathogenic HIV-1 Env encodes multiple pathogenic determinants including high fusion activity and pDC activation and IFN induction activity.

The HIV-1 Env plays a significant role for the pathogenicity of the virus. HIV-1 infection is characterized by continuous viral replication and depletion of the host immune cells. Although the host mounts a significant anti-viral response, HIV-1 achieves a persistent infection. Several factors are thought to contribute to HIV-1 viral persistence: the destruction of virus-specific T helper cells, the emergence of antigenic escape mutants, and the expression of an envelope complex that structurally minimizes antibody access to conserved epitopes (24). Various determinants of the HIV-1 Env allow for both a direct fusion-induced killing method as well as an indirect killing mechanism of uninfected cells.

It has been previously reported that co-receptor ligation can lead to bystander apoptosis (1, 22, 23), and that cell death can be mediated through Fas/Fas Ligand binding(2). Recently, Type 1 IFN has been proposed in a bystander cell death model based on clinical correlation in patients(18, 20). Plasmacytoid DCs are the primary IFN producing cells in the body(42), and it has been demonstrated that HIV-1 can activate pDC through endocytosis

that is CD4-mediated(4). Further, CD4 binding affinity has been suggested as the major determinant for pDC activation by HIV-1 Env(17). We report that the V1V2 (C2) domains of HIV-1 Env is the significant contributor to pDC activation and IFN induction, presumably due to the domain's role in elevated CD4 binding affinity(33). Interestingly, several amino acid differences exist between V1 and V2 domains of R3A and R3B that contribute to both CD4 binding affinity and pDC activation. Additionally, 2 amino acid differences exist within the C2 (constant domain 2) region that may contribute to this phenotype. Further analysis will be performed to determine the individual amino acid changes that confer R3A's enhanced phenotype.

During infection of HFTOC with NL-R3A, we observed that addition of C34 or T20 at the height of viral production would inhibit CD4+ depletion, while still allowing for high levels of HIV-1 production (36). We found that non-fusion mediated total cell depletion was inhibited significantly in the presence of IFNαβ neutralizing antibodies. This suggests that Type 1 IFN that provides an anti-viral response may also contribute to the depletion of CD4 T cells by HIV-1 infection.

The mechanism for this IFN mediated cell depletion remains unclear. It has been suggested that Type 1 IFN induces TRAIL ligand expression on target cells, which can then bind to the DR4/5 death receptors also induced on CD4 T lymphocytes, hence causing their death. Further experiments will be performed to determine whether this is indeed the pathway that leads to uninfected cell death by R3A infection. While it is suggested that pDCs may function as the TRAIL expressing killer cell (16), our preliminary data does not demonstrate a differential TRAIL expression on pDCs infected with R3A and R3B. Various populations of both lymphoid and non-lymphoid cells exist in the HFTOC environment, and

may express TRAIL that contributes to IFN-mediated cell depletion. Further analysis will be performed to determine the cell types that contribute to IFN-induced T cell depletion. Badley and colleagues have recently discussed the possibility of using TRAIL as a therapeutic, which could conceivably induce apoptosis in cells that serve as latent reservoirs during HIV infection(41). However, as it is now known that HIV particles (whether infectious or non-infectious), induce significant amounts of Type I IFN. If this IFN does indeed induce TRAIL-mediated apoptosis in both infected and un-infected cells, addition of exogenous recombinant TRAIL could exacerbate rather than ameliorate immune depletion and disease progression.

In this study, we find that HIV-1 induces Type 1 IFNs from plasmacytoid DCs through interaction with CD4 and endocytosis, and that IFNs have a pathogenic effect on CD4 T cells in the infected lymphoid organs. While there are many proposed mechanisms for bystander cell death during HIV-1 infection, it is interesting that in the absence of fusion, Type 1 IFN plays a significant role in CD4 T cell depletion. These data suggest that while HIV-1 fusion is a major contributor to pathogenesis *in vivo*, anti-IFN*a* as well as fusion inhibitors may be necessary to successfully protect CD4 T cell depletion due to HIV-1 infection.

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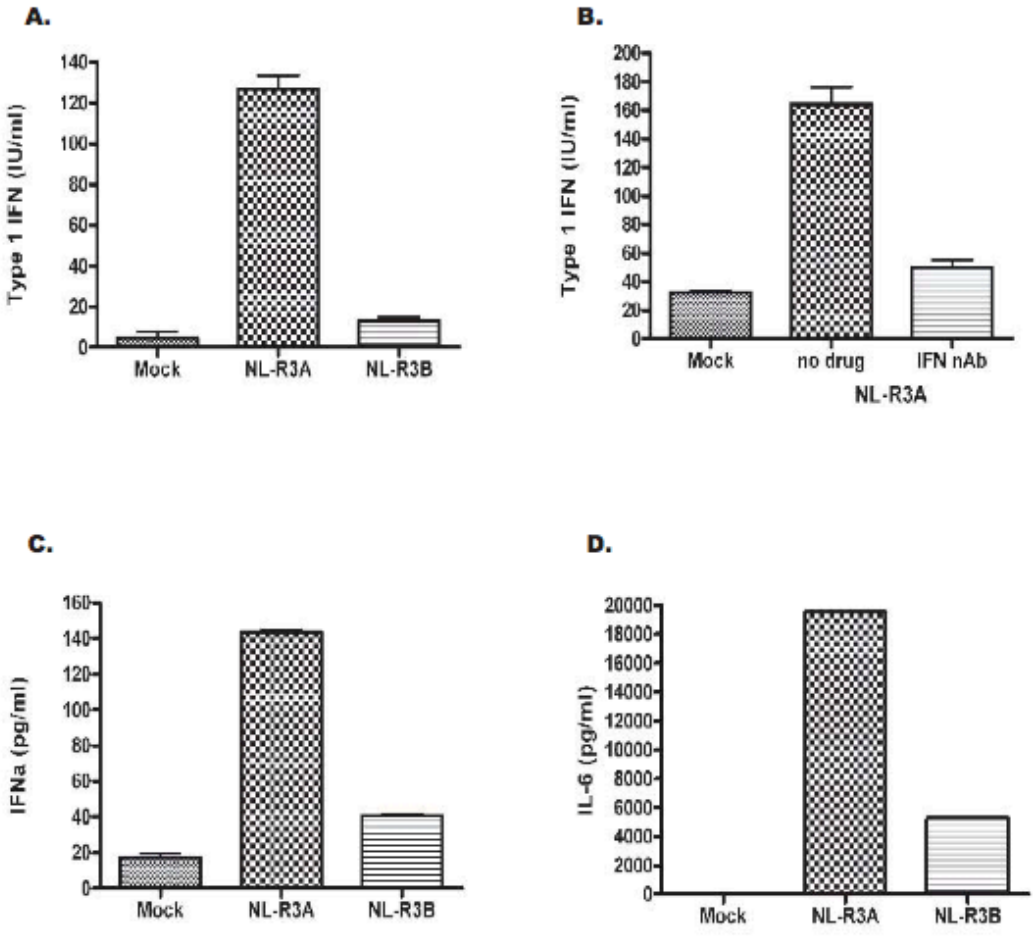
A.
Type 1 IFN (IU/ml)
140
120
100
80
60
40
20
0
Mock
NL-R3A
NL-R3B
B.
Type 1 IFN (IU/ml)
200
180
160
140
120
100
80
60
40
20
0
Mock
no drug
IFN nAb
NL-R3A
C.
IFNa (pg/ml)
160
140
120
100
80
60
40
20
0
Mock
NL-R3A
NL-R3B
D.
IL-6 (pg/ml)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Mock
NL-R3A
NL-R3B

Figure 1.

Figure 1: NL-R3A preferentially induced Type I IFN in PBMCs. A. NL-R3A induces ~10x more Type 1 IFN than N-R3B. Unstimulated PBMCs were infected with either NL-R3A or R3B for 12 hrs, and supernatants were analyzed for bioactive Type 1 IFN. **B**. Induction of IFNs by NL-R3A was significantly inhibited by IFNαβ nAb, demonstrating specificity of Bioassay for Type 1 IFNs. **C**. Induction of IFNα in resting PBMC by NL-R3A and NL-R3B was measured by ELISA (Bender). **D**. Expression of IL-6 by NL-R3A and NL-R3B was also measured by ELISA.

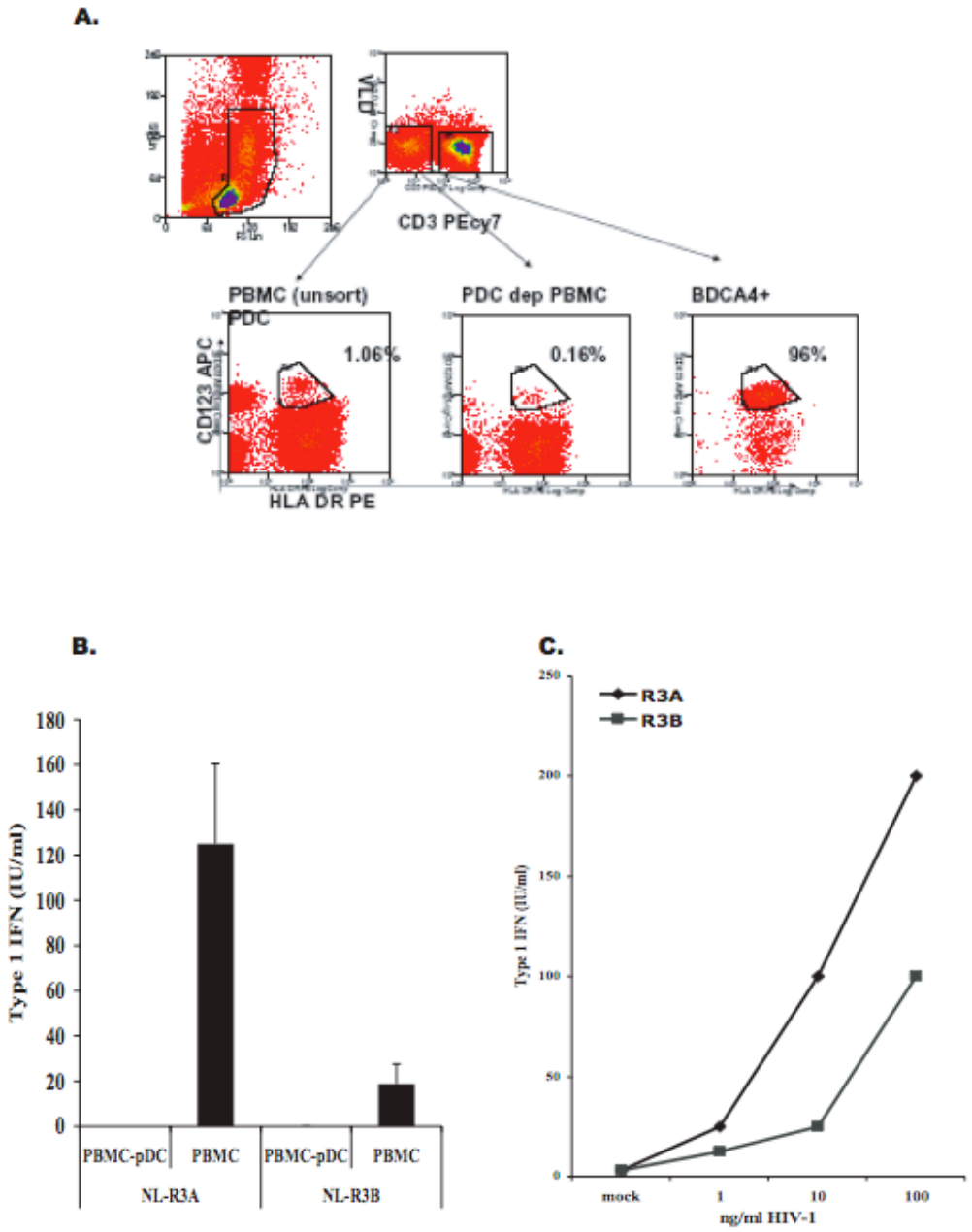
A.
CD3 PEcy7
PBMC (unsort)
PDC
PDC dep PBMC
BDCA4+
1.06%
0.16%
96%
CD123 APC
HLA DR PE
B.
Type 1 IFN (IU/ml)
180
160
140
120
100
80
60
40
20
0
PBMC-pDC
PBMC
PBMC-pDC
PBMC
NL-R3A
NL-R3B
C.
R3A
R3B
Type 1 IFN (IU/ml)
250
200
150
100
50
0
mock
1
10
100
ng/ml HIV-1

Figure 2.

Figure 2. R3A interacts with pDC to induce IFNs. A. Fresh, unstimulated PBMCs were sorted for pDC, with purity of >95%. **B.** Neither NL-R3A or R3B induced IFN is pDC depleted cells, but differential expression is seen in whole (un-sorted PBMCs). **C.** Differential IFN induction is seen with purified pDC, and this induction is viral dose dependent.

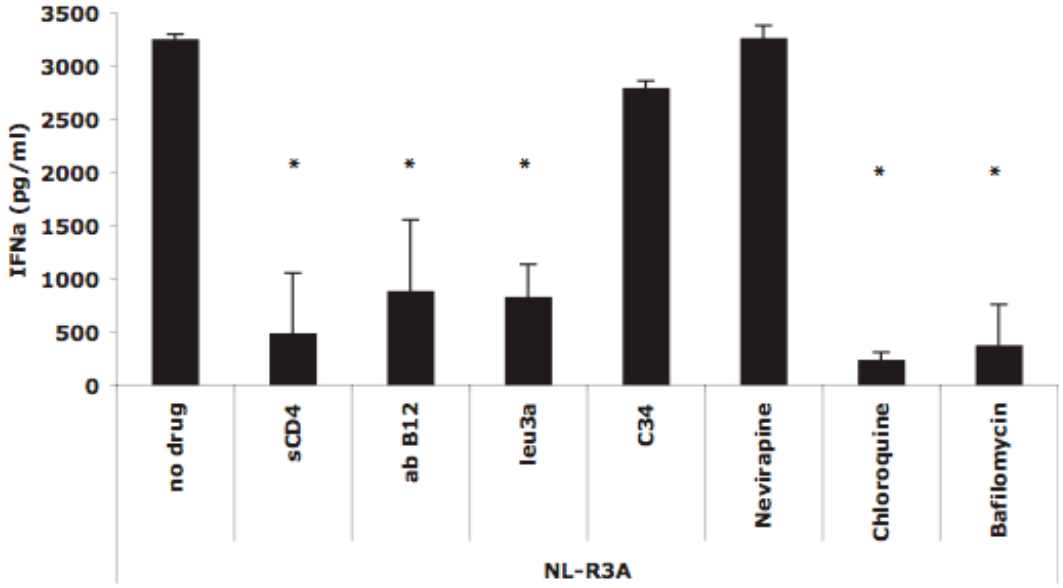
IFNa (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
*
*
*
*
*
no drug
sCD4
ab B12
leu3a
C34
Nevirapine
Chloroquine
Bafilomycin
NL-R3A

Figure 3.

Figure 3: HIV induction of IFN is not impeded by Fusion or RT inhibitors. NL-R3A induces high amounts of IFNα in fresh unstimulated PBMCs in the presence of fusion inhibitor (C34) and RT Inhibitor (Nevirapine), but not inhibitors of CD4 (sCD4, Leu-3a, or B12 antibody) or endocytosis (Chloroquine, Bafilomycin A1). * indicates significance over "no drug", with $p < 0.001$.

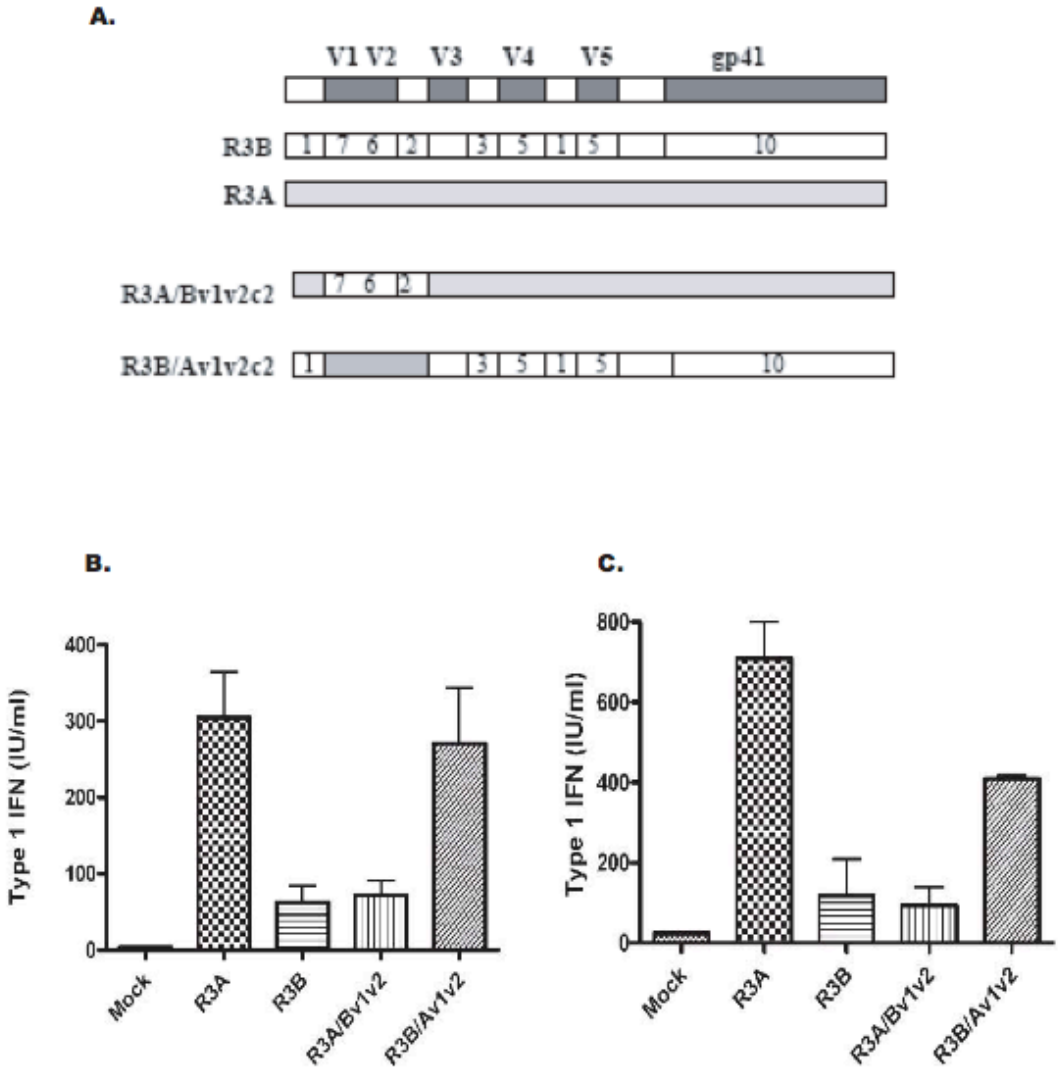
A.
V1 V2
V3
V4
V5
gp41
R3B
1 7 6 2 3 5 1 5 10
R3A
R3A/Bv1v2c2
7 6 2
R3B/Av1v2c2
1 3 5 1 5 10
B.
Type 1 IFN (IU/ml)
400
300
200
100
0
Mock
R3A
R3B
R3A/Bv1v2
R3B/Av1v2
C.
Type 1 IFN (IU/ml)
800
600
400
200
0
Mock
R3A
R3B
R3A/Bv1v2
R3B/Av1v2

Figure 4.

Figure 4: The HIV Env V1V2 domains confer Type 1 IFN induction in pDC. A. Full-length V1V2 Chimeras were developed between NL-R3A and R3B. **B/C**. NL-R3A, R3B and chimeras cultured with both unstimulated PBMC (**B**) and sorted pDC (**C**). Supernatants were harvested 12 hours post infection, and analyzed for Type IFNs by IFN bioassay. Error bars demonstrate standard deviation with n=3.

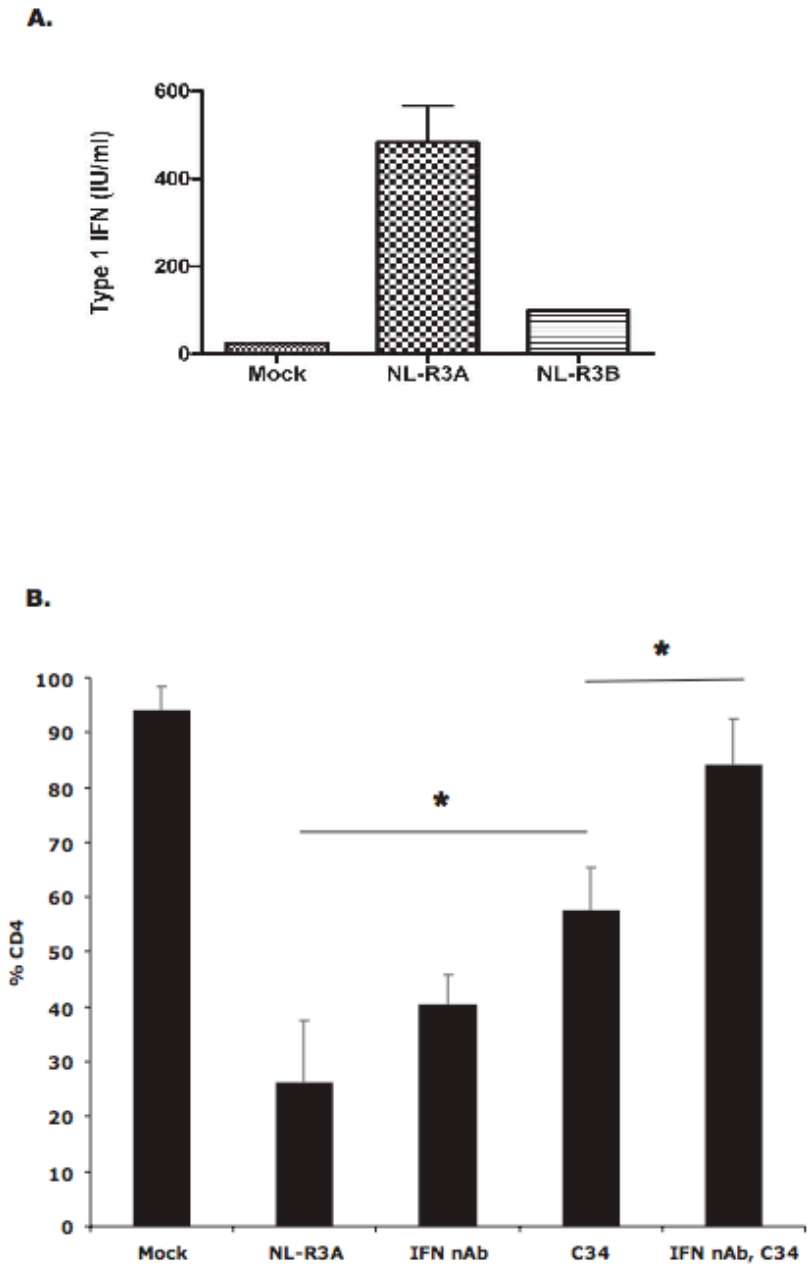
A.
Type 1 IFN (IU/ml)
600
400
200
0
Mock
NL-R3A
NL-R3B
B.
% CD4
100
90
80
70
60
50
40
30
20
10
0
*
*
Mock
NL-R3A
IFN nAb
C34
IFN nAb, C34

Figure 5.

Figure 5: HIV-1 induced Type 1 IFN significantly contributes to thymocyte depletion. A. IFN expression by NL-R3A and NL-R3B was measured from HIV-1 infection in HFTOC. Supernatants were havested 24hrs after infection for detection by Bioassay. **B.** HFTOC was infected NL-R3A, in the presence or absence of anti-IFNαβ, C34, or both anti-IFNαβ and fusion inhibitor. Drugs were added 5 dpi, and HFTOC was harvested 8 dpi for FACs analysis of depletion. Error bars indicate standard deviation with n=3, and * < 0.05 indicates significance.

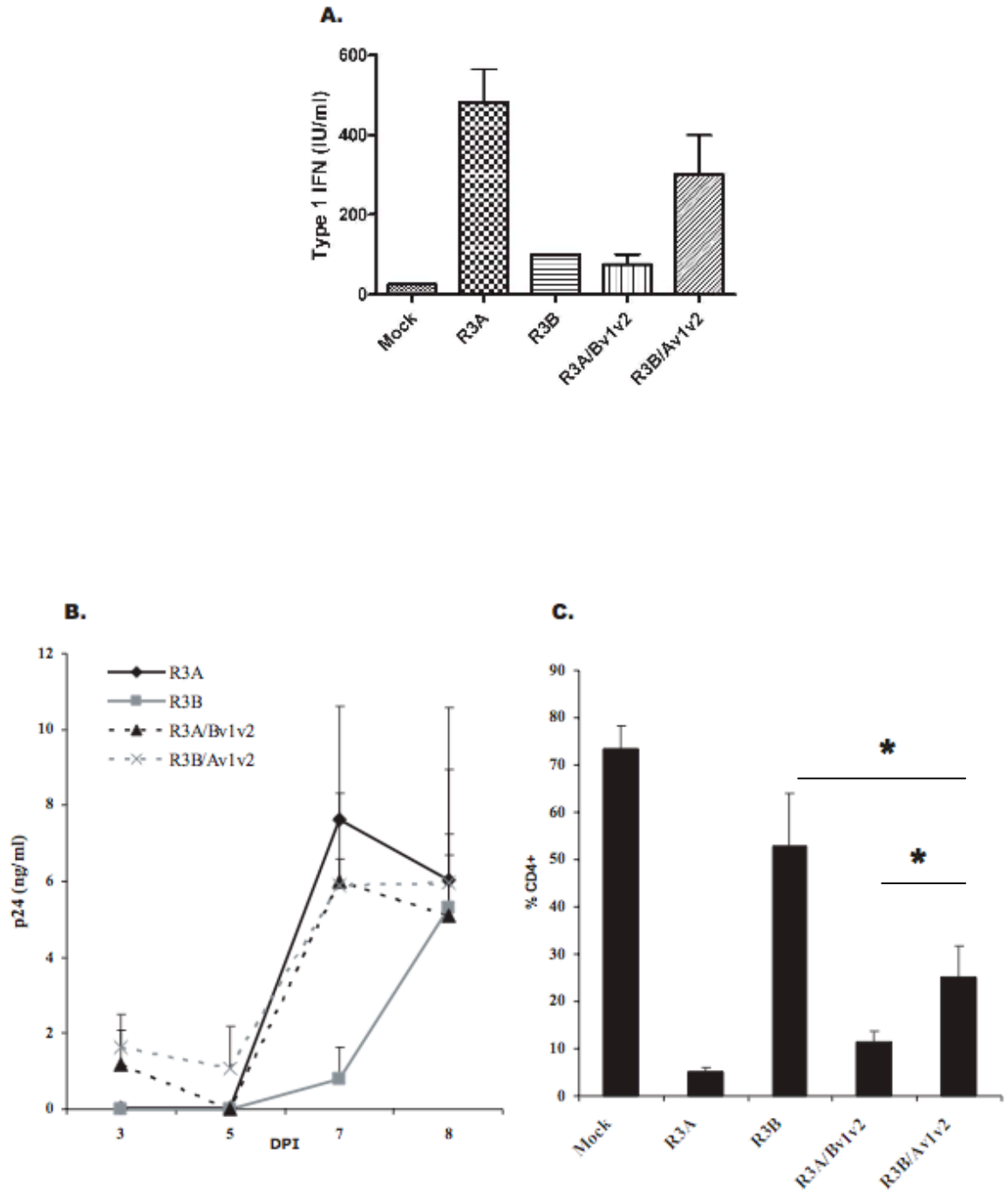
A.
Type 1 IFN (IU/ml)
600
400
200
0
Mock
R3A
R3B
R3A/Bv1v2
R3B/Av1v2
B.
p24 (ng/ml)
12
10
8
6
4
2
0
R3A
R3B
R3A/Bv1v2
R3B/Av1v2
3
5
7
8
DPI
C.
% CD4+
90
80
70
60
50
40
30
20
10
0
*
*
Mock
R3A
R3B
R3A/Bv1v2
R3B/Av1v2

Figure 6.

Figure 6: The R3A V1V2 domain confers both enhanced IFN induction and pathogenesis in HFTOC. A. HFTOC fragments were infected with NL-R3A, R3B and chimeras. Supernatants were harvested 24 hours post infection, and analyzed from Type 1 IFNs by IFN Bioassay. Error bars indicate standard deviation with n=3. **B/C**. HFTOC was infected with NL-R3A, R3B and chimeras. Viral replication (**B**) was measured by p24 ELISA and CD4+ thymocyte depletion (**C**) was measured by FACS analysis at 8 dpi. *<0.05.

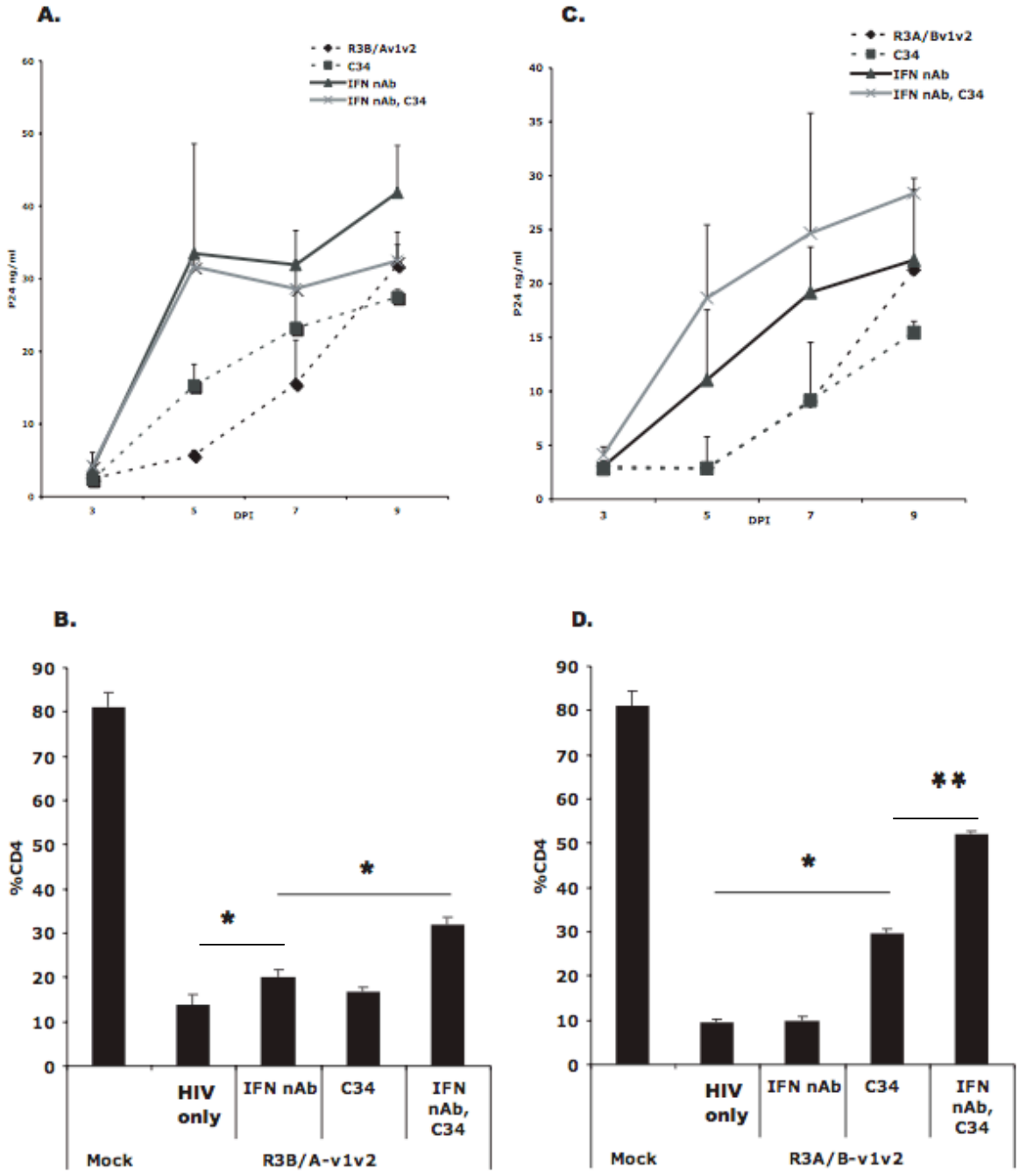
A.
R3B/Av1v2
C34
IFN nAb
IFN nAb, C34
P24 ng/ml
DPI
C.
R3A/Bv1v2
C34
IFN nAb
IFN nAb, C34
P24 ng/ml
DPI
B.
%CD4
HIV only
IFN nAb
C34
IFN nAb, C34
Mock
R3B/A-v1v2
D.
%CD4
HIV only
IFN nAb
C34
IFN nAb, C34
Mock
R3A/B-v1v2

Figure 7.

Figure 7: The R3A V1V2 domain contributes to IFN-mediated death in HFTOC. HFTOC was infected with NL-R3B/A-v1v2 (**A**) or NL-R3A/B-v1v2 (**B**), in the presence or absence of anti-IFNαβ, C34, or both drugs. Anti-IFNs were added on 0 dpi to thymic fragments, while C34 was added at 5dpi. HFTOC was harvested at 9 dpi to FACS analysis of depletion. Error bars indicate standard deviation with n=2, and * < 0.05, ** < 0.005.

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Chapter 4

HIV-1 Nef protein Enhances HIV Env to Activate Plasmacytoid DC through a CD4-Dependent Pathway.

(Work still in progress)

Abstract:

The Nef protein of Human Immunodeficiency Virus-1 (HIV-1) enhances both viral infectivity and pathogenesis in many models of infection, although the mechanisms remain unclear. It is becoming increasingly evident that during HIV infection, Type 1 interferons (IFNs) may play a pathogenic role on uninfected cells. We (and others) have previously reported the functional significance of HIV Env to induce Type 1 IFN through activation of plasmacytoid dendritic cells (pDCs) and the resulting capacity to deplete CD4+ cells in a fusion-independent manner. Here, we find that the Nef protein is also required for efficient Type 1 IFN induction by HIV-1 from pDCs. Interestingly, Nef-mediated pDC activation is dependent on CD4-expression on the cell type that produces the virion. We propose that downregulation of CD4 provides for enhanced viral expression of HIV-1 Env, as a mechanism for Nef-mediated pDC activation (and Type 1 IFN induction) during HIV-1 infection.

Introduction:

Type 1 Interferons (IFNs) are known to play an important role in both the innate and adaptive immune response against invading viral pathogens. The host immune response has a vsriety of methods to detect viral pathogens, and quickly responds with a release of interferon αβ. The secreted proteins then bind to neighboring cells through IFN αβ receptors, thus amplifying the secretion of proteins and progressing the cells into an anti-viral state. This state is characterized by the activation of a large number of Interferon-Stimulated Genes (ISGs), as well as curbing viral replication. The fact that most pathogenic viruses have developed methods to evade detection of the IFN system demonstrated the significant anti-viral capacity of these proteins.

Evidence that Type 1 IFNs offer therapeutic benefit during HIV-1 infection is based on *in vitro* analysis, and currently debated for clinical use(17, 23, 24). In fact, the level of IFNs present in serum of HIV-infected individuals is a clinical monitor of disease, such that decrease in IFN levels correlate with disease progression. Recently, pDCs and Type 1 IFNs have been implicated in bystander cell death during HIV-1 infection(9, 10). PDCs are the major Type 1 IFN-producing cell *in vivo*, and are regarded as the first line of defense against invading viral pathogens. Although they can be productively infection through the receptors CD4, CXCR4 and CCR5, pDC are known to respond to HIV-1 by first up-taking HIV-1 through CD4 receptor-mediated endocytosis(5, 22). While in the endosome, TLR7 detection of HIV RNA is thought to signal the rapid induction of IFNαβ. We (and others) have shown that CD4 binding affinity (mediated by the V1V2 domains of HIV Env) determine the ability

for HIV to induce IFN from pDC(8, 14). Furthermore, we have recently shown that this induction of IFN promotes bystander cell death in a relevant organ model of infection.

The Nef protein is a significant virulence factor in primate lentiviruses. The Nef gene encodes for a 27-34 kd protein that is myristoylated at the amino terminus, and has been shown to bind to many cellular proteins that may aid in its role in pathogenicity(1, 2, 13, 21). Though not required for infectivity, this accessory protein plays a significant role in development of disease(4, 6, 7). The importance of Nef in HIV-1 pathogenicity is further highlighted by reports that a subset of long term non-progressors are infected with Nef-defective HIV-1, and that expression of Nef in mouse models induces pathology similar to human AIDs disease(3, 11, 18, 20).

We find that (along with HIV-1 Env), the Nef protein is required for IFN induction *in vitro*. Further, the induction of IFN by HIV-1 is specific to CD4 expression on the cell that derives the virus. By genetically mapping the functional domains within the Nef gene that may play a dominant role in HIV-induction of Type 1 IFNs from pDC (in resting PBMC), we expect to develop a mechanism for Nef-dependent pDC activation. We believe that this analysis will identify a novel role for Nef in HIV-1 mediated pathogenesis.

Materials and Methods:

Cells and Plasmids. A293T cells were maintained in Dulbecco's Modified Essential Media (Invitrogen) supplemented with 10% (vol/vol) bovine calf serum (Sigma Chemical, St. Louis, Mo.) and 100 μg of penicillin and streptomycin/ml. All viral isolates were prepared and stored in Iscove's Modified Dulbeccos Media supplemented with 10% (vol/vol) bovine calf serum and 100 μg penicillin and streptomycin/ml. Peripheral blood monocytes were purified from the blood of healthy HIV-1 negative donors by Ficoll-Plaque density gradient centrifugation and cultured in Iscoves Modified Dulbeccos Media supplemented with 10% bovine calf serum and 100 μg penicillin and streptomycin/ml. HSCG-huCD4(short) is a Human-CD4 expressing plasmid that contains a truncation in the cytoplasmic domain that allows for enhanced surface expression on cells, and was generated by in the Su lab.

Viral production. Full-length infectious viral DNA were transfected into A293T, and then supernatant was harvested at 48 hrs post transfection, and co-cultured with PHA/IL-2 stimulated PBMCs. NL4.3nef mutant proviral DNAs were obtained as a kind gift from Dr. J. Zack and from Dr. C. Aiken.

Viral Quantitation. Gag was detected in viral stocks as well as supernatant from HFTOC infections using a p24 enzyme-linked immunosorbent assay (ELISA) kit (AIDS Vaccine Program, NIH). Viruses were quantified for infectious units/ml by infection of Magi (CXCR4) cells, as previously reported.

Human Fetal Thymus Organ Culture (HF-TOC): The procedure for infection and culture of human fetal thymus has been previously described (14-16). In brief, human fetal thymuses

(19 – 22 weeks of gestation) were dissected into ~ $2mm^2$ fragments, using a dissecting microscope to retain the lobe structure for each fragment.

Four fragments were placed on organotypic culture membranes (Millipore) and under-laid with HFTOC media (previously described) were plated in a 6 well tissue culture plates. Thymic fragments were infected with equivalent amounts of virus (100-800 IU) in 15 ul per fragment. Viral and mock supernatants were derived from the same donor sample per experiment.

Thymic fragments were cultured at 37°C in 5% $C0_2$ for the length of each experiment. At harvest day of experiments, thymic fragments were disassociated in 350ul PBS with 2% FBS using pestels (Bellco Co.) for Florescent-Activated Cell Sorting (FACS) Analysis.

FACS Analysis. Thymocytes were harvested and stained for CD3+, CD4+, CD8+ and viability using Fluorescence labeled antibodies (Caltag and Invitrogen) and analyzed on a Cyan Fluorescence Cytometer, using Summit software.

Interferon Analysis. As reported earlier, resting PBMCs were infected with NL-R3A, NL4.3 and various mutants at 10-100 ng/ml p24 for 12-16 hours. Supernatants were harvested, acidified (to removed infectious HIV-1) and analyzed for Type 1 IFN expression using an IFN Bioassay and IFNα ELISA (Bender Systems). The IFN Bioassay was performed as follows: Supernatants harvested from infections were over-layed on A549 cells. 24 hours later, interferon-sensitive EMCV was added to A549 cells. After 18hrs, remaining viable cells (due to protection by IFNα/β in the supernatant) were quantified in comparison to known IFN Standard (NIH AIDS Research and Reference Reagent Program). Viable cells were removed from plated by Trypsin Reagent (Sigma Chemical, St. Louis and quantified using Guava Easycyte Analysis (Guava Technologies)

Results And Future Directions:

HIV-1 requires Nef protein expression for IFN Induction in un-stimulated PBMCs.

Our laboratory has previously shown that the V1V2 domains of HIV-1 Envelope (Env) are a chief determinant for virion-induced Type 1 IFN induction from pDCs. These studies were initiated to determine factors that allow for the enhanced pathogenicity of viral isolate obtained from a rapid progression patient from the ALIVE cohort, denoted as R3A. Initial cloning of the Env into common NL4.3 backbone functionally deleted the NL4 Nef expression, but still yielded a significantly pathogenic Env due to its capability for enhanced fusogenicity. However, when the NL4 Nef expression was repaired in this Env, a more robustly pathogenic viral Env was generated. Human fetal Thymus Organ Culture (HF-TOC) was infected with equivalent titers of NL-R3A(-Nef) or R3A(+Nef), and depletion of CD4+ thymocytes was measured at various days post infection (**Fig 1**). At 13 days post infection with R3A(-Nef), a ~75% depletion of thymocytes was observed. However, at 11 days post infection with R3A(+Nef), an ~80% depletion of CD4+ thymocytes was observed (**Fig 1a**). CD4+ thymocyte depletion by NL-R3A(+Nef) was enhanced in time course and severity, compared to depletion by NL-R3A(-Nef).

Interestingly, inhibition of fusion at a time of high viral load almost completely protected thymocytes from depletion by R3A(-Nef), in both percentage as well as in total number of CD4+ cell depletion (**Fig 1b**). However, depletion of CD4+ cells by R3A(+Nef) was significant even after fusion-inhibition. As we have previously demonstrated that Type 1 IFN contributes to cell death of uninfected (non-fused) cells, we compared Type 1 IFN induction ability between the two viruses. Resting PBMCs were infected with NL-R3A(+/-

Nef), and NL4.3 (+/- Nef) at equivalent titers for 12 hrs. Bioactive Type 1 IFN was detected in supernatants harvested early in infection by IFN Bioassay (**Fig 2**). These data demonstrated significant induction of IFNs by viruses expressing Nef, but not by Nef-deleted viruses.

Nef-Mediated IFN Induction is specific to PBMC-Derived virus.

To generate virus, proviral DNA is transfected into A293T to obtain newly packaged virion for amplification in PBMCs. The viral stocks used for infection are generated from short-term amplification of virus using activated PBMCs, to represent a biologically relevant virus found *in vivo*. We compared IFN induction ability between PBMC-derived and A293T-derived virus. Resting PBMCs were infected with either PBMC-derived R3A+/- Nef virus or A293T-derived viruses for 12 hrs. Supernatants were harvested and analyzed by IFN Bioassay (**Fig 3**). These data suggest that Nef-mediated IFN induction (that was previously observed) is specific to PBMC-derived virus. Robust expression of Type 1 IFN was found from infection of resting PBMCs with PBMC-derived R3A(+Nef), but not R3A(-Nef). Virus derived from A293T cells, however, induced markedly less IFN (slightly above the limit of detection) that was not observed to be Nef-specific. These results suggest that virus derived from different cell types may display different activation phenotypes upon resting PBMCs. As amplification of virus within activated PBMCS (3-8 days post infection) is not found to induce mutations in virion (data not shown), differences in virion-associated surface proteins may play a role in this phenotype.

Cell Surface Expression of CD4 plays a role in HIV+Nef mediated-IFN Induction.

A293T cells are of epithelial origin where as PHA/IL-2 activated PBMCs express mainly lymphoid cells, so many different cell receptor expression profiles may play a role in this cell-activation phenotype. However, CD4 binding affinity was previously shown to determine IFN production on pDCs by HIV-1, so CD4 expression may have a role in the observed phenotype of Nef-mediated differential IFN induction. To test this hypothesis, A293Ts were co-transfected with HIV(+/-Nef) and a plasmid construct that expresses human CD4 on cell surface. Two days after transfection, cells were stained for CD4 and HIV-1 Env expression (2G12) and analyzed by FACS. Relatively high CD4 expression was observed on cells either with no HIV-1 or with transfection of HIV(-Nef) (**Fig 4a**). Interestingly, HIV Env expression was enhanced in cells co-transfected with HIV(+Nef) compared to HIV(-Nef) (**Fig 4b**). Although CD4 down-regulation was observed upon cells transfected with HIV(+Nef) compared to HIV(-Nef), but the differences were not as robust as expected. Distinct CD4 down-regulation was not observed due to Env expression, and may be due to deletions within the cytoplasmic tail of the CD4-expressing plasmid, which has been previously suggested to be necessary for Nef mediated down-regulation(19).

Resting PBMCs were infected by equivalent titers of viral supernatants for 12 hrs. Supernatants were harvested, and we detected Type 1 IFN by IFNα ELISA (**Fig 4c**). Virus derived from co-transfection of CD4-expressing plasmid with HIV(+Nef) induced robust IFNα expression in resting PBMCs, while no IFNα was detected by infection with Nef-deleted virus. Nef-mediated down-regulation on virus producing cells may produce virus more apt to induce Type 1 IFN in pDCs found in resting PBMCs. Transfection of A293T

cells with HIV(+/-Nef) in the absence of CD4 co-transfection demonstrated relatively poor Env expression on the surface of cells. Furthermore, virus generated from these cells induced significantly attenuated amounts of IFNα from resting PBMCs (**Fig 4d**). These data suggest that CD4 expression on virus-producing cells plays an important role in IFN inducing potential of HIV+Nef viruses.

In concert with findings reported by Lundquist et al**,** we believe that Nef functions to down-regulate CD4 expression in CD4+ lymphocytes, thus allowing for more HIV-1 Env expression on the cell surface, and hence on the virion surface(12). **We hypothesize that enhanced Env expression on virion will allow for more ENV/pDC interaction, and hence more pDC activation.** To address this hypothesis, several experiments must still be performed:

1) Identify HIV Expression on Virion Surface.

We will perform Western-blot analysis for HIV Env on HIV+Nef virus produced from A293T +/- CD4. We expect that ENV expression will be enhanced on HIV+Nef virion that is derived from transfection of A293T+CD4, compared to virus derived from A293T in the absence of CD4 expression. We also expect there will be significantly lower ENV expression on HIV-Nef, in both cells, +/- CD4 expression.

2) **Identify Domain within Nef that plays a role in the IFN Induction Phenotype.**

We have obtained several NL4.3 viruses carrying mutations in various domains of the Nef gene. These mutants have been previously reported to effect different known cellular binding proteins, down-regulation of MHC class I and CD4. We plan to utilize these mutants

to map the domain(s) necessary for IFN induction of A293T+CD4 derived virus, in an attempt to understand the function that plays in this phenotype. We hypothesize those mutations that effect CD4 down-regulation will also negatively affect IFN induction ability of virus.

We believe that these experiments will distinguish the domain(s) of Nef required for IFN induction as well as provide a mechanism for enhanced Env-mediated IFN induction observed in resting PBMCs.

Discussion:

The HIV-1 Nef protein participates in several known cell interactions, but the protein's role in HIV-1 pathogenesis remains unclear. Nef is clearly a significant pathogenic factor for HIV-1, in that deletion of Nef has generated attenuated HIV-1 virus in both *in vitro* and *in vivo* infection models. We find that HIV-1 Nef is required for efficient activation of pDCs. This phenotype is dependent on human CD4 expression on the surface of virus-producing cells. Interestingly, the Nef protein may also play a role in pDC exhaustion against further stimulation by TLR9 agonists. By inducing a somewhat conflicting response, the Nef protein may contribute initially to the innate immune activation phenotype during initial infection, but then may also contribute the lowering of this activation for the onset of chronic infection.

The HIV-1 Nef protein is shown to perform many functions and interaction with the host cell during infection. Much work has been performed to characterize the domains of interest that function to induce MHC I down regulation, ASK and PAK2 signaling, CD4 down regulation as well as enhancing viral replication. It has even been postulated that the inability of HIV-1 Nef to down regulate CD3 may significantly contribute to pathogenesis by HIV-1. We believe that we present a novel function of HIV-1 Nef to work (in conjunction with HIV Env) to activate pDCs and induce fusion-independent cell death. This pathway likely requires the CD4 endocytosis pathway as a method to enhance HIV Env on the surface of virion. When these virions then engage pDCs, enhanced activation has been shown to induce fusion-independent death in human fetal thymus organ culture. The relevance to this finding is found in fact that HIV01 primarily infects and replicates in CD4+ T cells,

demonstrating a reliance of a viral protein on a host protein for infectivity. This lends deference to the idea of co-evolution of viral proteins and host cells to produce an initially pathogenic effect in the host.

Several experiments must be completed to develop a clear understanding of mechanism for this finding. First, protein analysis for HIV Env must be performed upon virions derived from A293T(+CD4) cells (+/-Nef) to test our hypothesis that Nef-mediated CD4-downregulation enhances Env-expression on virus-surface. We can compare these results to virus derived from activated PBMCs, as well as virus derived from wild type A293T. Second, we will identify the domains within the Nef protein that contribute to pDC activation, using mutants obtained from collaborators. Isolates from both the Aiken and Zack labs that carry various mutations and deletions within the Nef gene will be used to characterize this phenotype. Finally, our group has initial data suggesting a role for Nef in inactivation or exhaustion of pDCs. This phenotype will be studied, from both the side of the virus and the host cell. We will utilize our Nef mutant viruses to characterize the viral domains relevant for this phenotype as well as signaling factors within the cells that pay a role in this response.

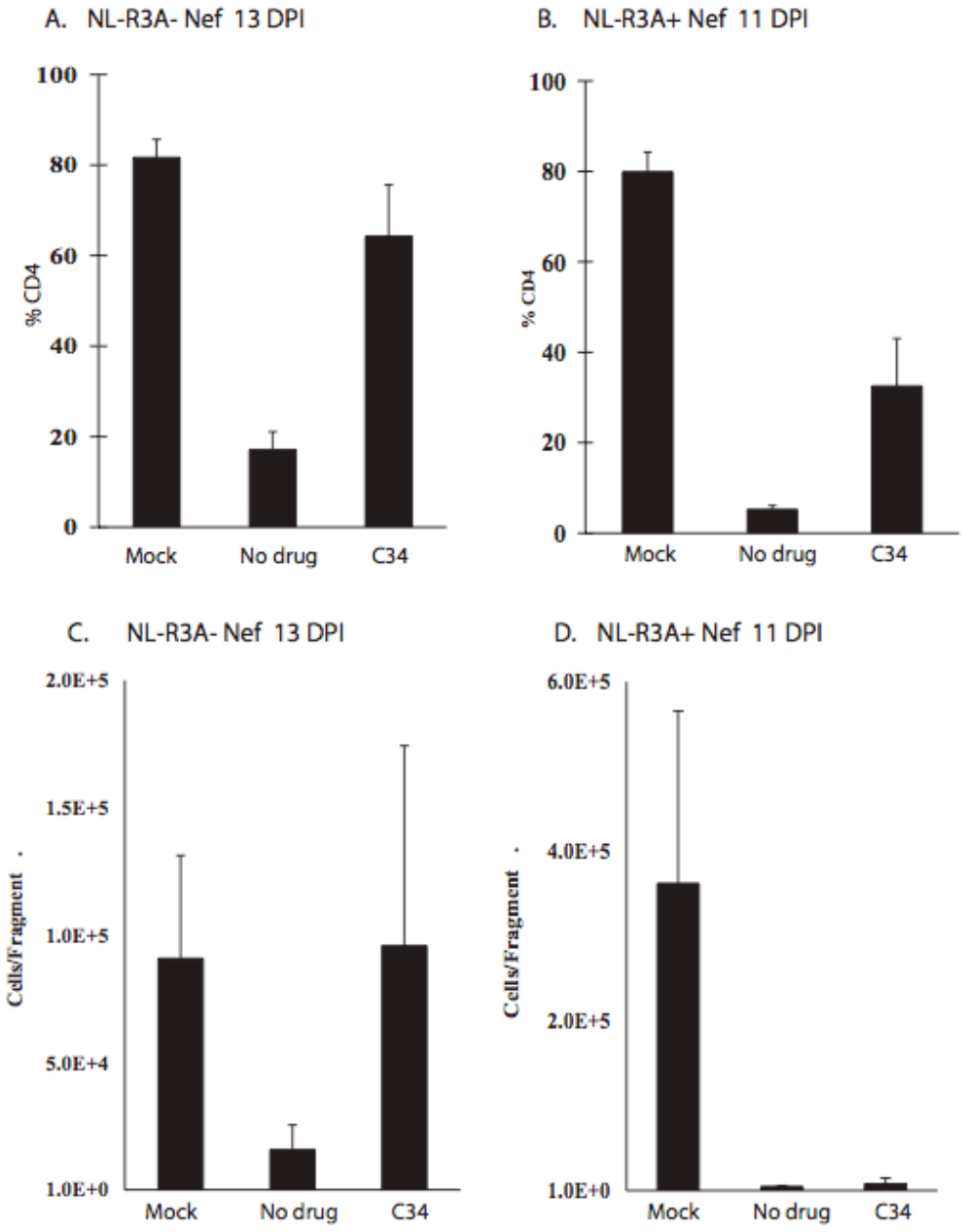
A. NL-R3A- Nef 13 DPI
100
80
60
40
20
0
% CD4
Mock
No drug
C34
B. NL-R3A+ Nef 11 DPI
100
80
60
40
20
0
% CD4
Mock
No drug
C34
C. NL-R3A- Nef 13 DPI
2.0E+5
1.5E+5
1.0E+5
5.0E+4
1.0E+0
Cells/Fragment
Mock
No drug
C34
D. NL-R3A+ Nef 11 DPI
6.0E+5
4.0E+5
2.0E+5
1.0E+0
Cells/Fragment
Mock
No drug
C34

Figure 1.

Figure 1: HIV+Nef depletes CD4+ thymocytes at a heightened pace compared to HIV-Nef. HFTOC was infected with R3A+/-Nef, and CD4+ thymocytes were measured for depletion at various days post infection. Fusion inhibitor (C34) was added at time points of high viral load to measure non-fusion dependent cell depletion. **A,B.** Depletion of CD4+ T cells by NL-R3A-/+Nef was measured by Flow Cytometry analysis. **C,D.** Depletion of total thymocytes due to infection by NL-R3A+/-Nef was quantified by Guava Analysis. Error bars reflect standard deviation with n=3.

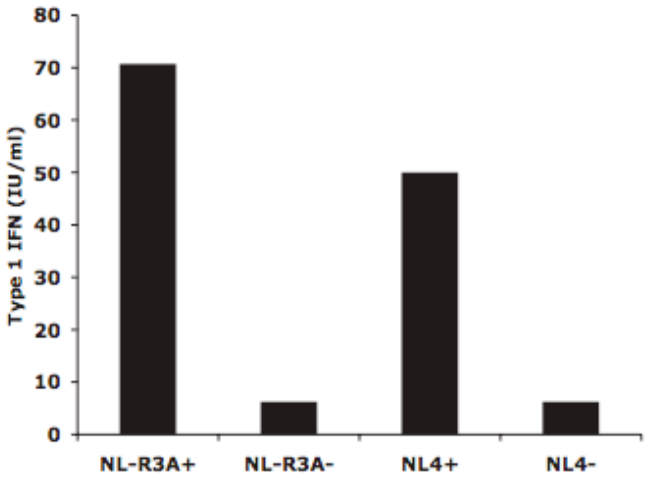

Figure 2.

Figure 2: Interferon Induction in Resting PBMCs requires Nef expression. HIV expressing different ENVs +/-Nef and were used to infect resting PBMCs at equivalent titers for 12 hrs. Supernatants were analyzed for Type 1 IFN by Bioassay.

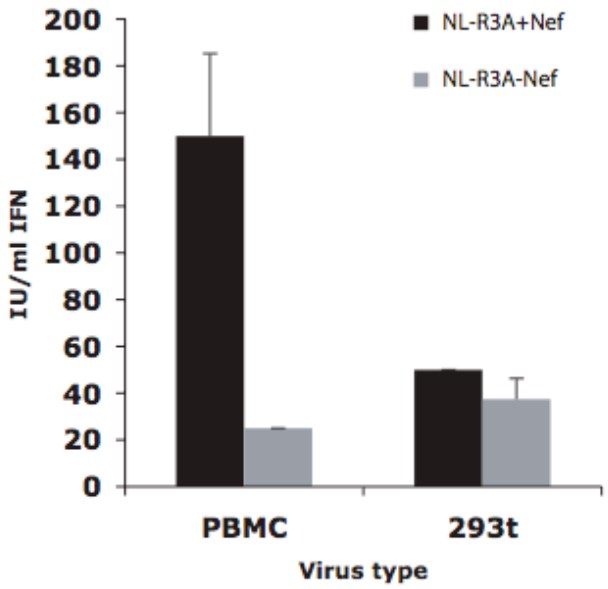
200
180
160
140
120
100
80
60
40
20
0
IU/ml IFN
NL-R3A+Nef
NL-R3A-Nef
PBMC
293t
Virus type

Figure 3.

Figure 3: The type of cells used to generate virus determines Nef-mediated IFN induction. Viruses were generated by either transfection of proviral DNA into A293T or by passage of virus in activated (PHA/IL2) PBMCs. Resting PBMCs were then infected for 12 hrs with HIV+/-Nef derived from either cell type, and supernatants were harvested from IFN bioassay. Error bars reflect standard deviation with n=2.

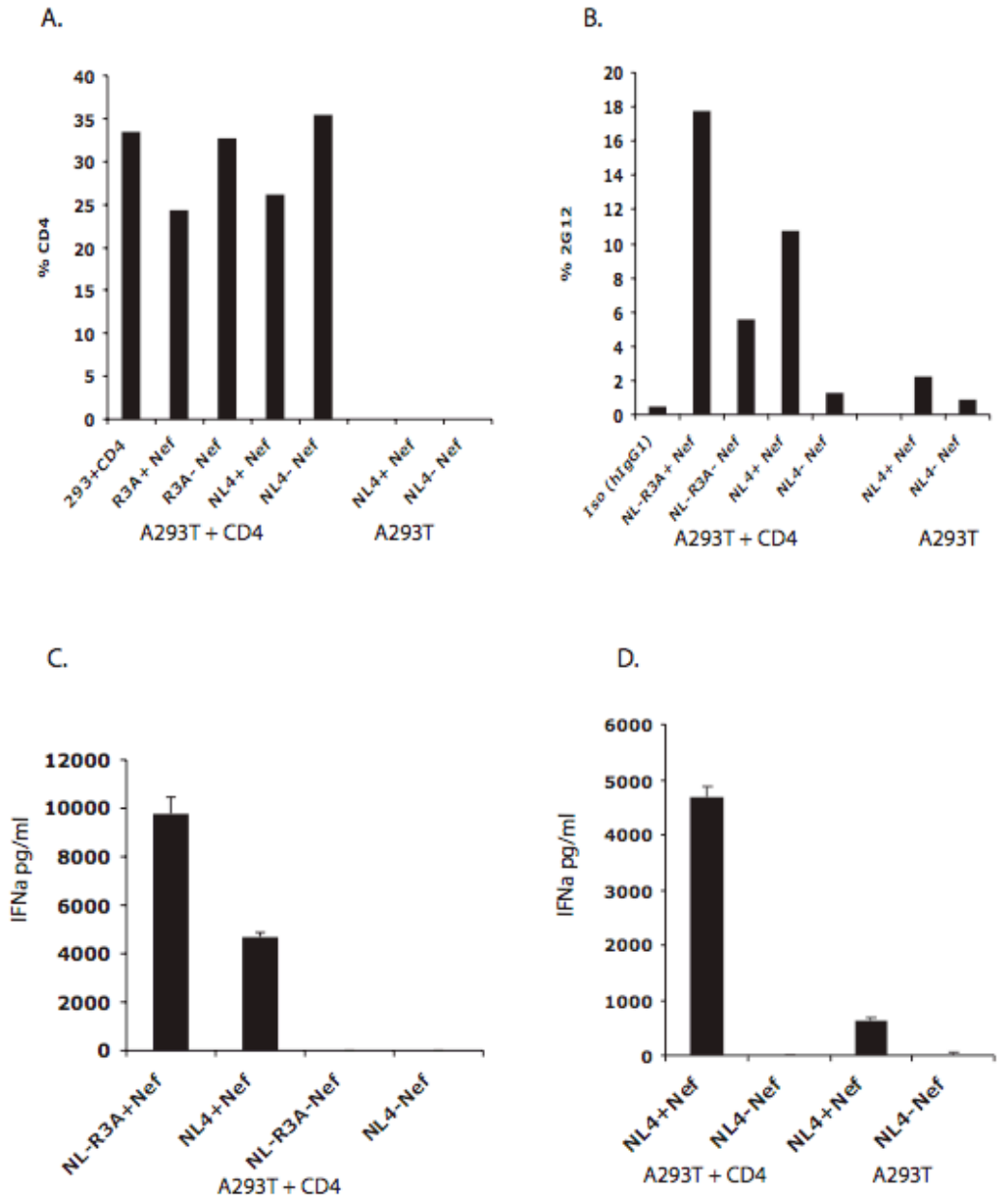
A.
% CD4
40
35
30
25
20
15
10
5
0
293+CD4
R3A+ Nef
R3A- Nef
NL4+ Nef
NL4- Nef
NL4+ Nef
NL4- Nef
A293T + CD4
A293T
B.
% 2G12
20
18
16
14
12
10
8
6
4
2
0
Iso (hIgG1)
NL-R3A+ Nef
NL-R3A- Nef
NL4+ Nef
NL4- Nef
NL4+ Nef
NL4- Nef
A293T + CD4
A293T
C.
IFNa pg/ml
12000
10000
8000
6000
4000
2000
0
NL-R3A+Nef
NL4+Nef
NL-R3A-Nef
NL4-Nef
A293T + CD4
D.
IFNa pg/ml
6000
5000
4000
3000
2000
1000
0
NL4+Nef
NL4-Nef
NL4+Nef
NL4-Nef
A293T + CD4
A293T

Figure 4.

Figure 4: Both CD4 cell surface expression and Nef expression in virion play roles in IFN induction by HIV. A. Relatively equivalent CD4 expression was found on CD4-transfected cells, but not un-transfected cells. A293T cells were co-transfected with CD4-expressing plasmid and plasmids expressing full-length HIV+/-Nef. %CD4 expression was analyzed on cell surface by FACS analysis. **B.** HIV Env is downregulated on cells co-transfected with CD4 and HIV-Nef. HIV Env expression was observed using 2g12 anti-HIV Env antibody staining and FACS analysis. **C.** Co-tranfection of CD4 and HIV+Nef produced virus that induces IFN from resting PBMCs. Equivalent titers of virus obtained from A293T transfection were used to infect resting PBMCs for 14hrs. Supernatants were analyzed by ELISA for IFNα. **D.** HIV induction of IFN is both Nef and CD4 dependent. Supernatants from transfected cells were used to infect resting PBMCs for 14hrs. Error bars reflect standard deviation with an n=3.

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Chapter 5

Conclusions and Future Directions

The purpose of this course of study is to further elucidate the complex nature of the interplay between HIV-1 and the human immune system. Through a detailed study of viral pathogenesis, I believe that mechanisms of pathogen/host interaction can be determined. Herein, I have described three studies that attempt to gain a better understanding of both viral- and immune-mediated factors that contribute to cell death. First, I utilized a mutant HIV-1 (that lacks Nef expression) to study direct cell death observed in a relevant lymphoid microenvironment. HIV-1 Env-mediated fusion to the host cell membrane (but not CXCR4 binding efficiency) was found to be the most dominant factor that allowed for enhanced pathogenesis by the R3A Env. Second, a bystander killing phenotype was observed with the re-introduction of Nef into R3A Env, and this was found to be fusion independent. In an effort to elucidate this pathway of cell death, activation of pDC by HIV-1 (in an Env-dependent fashion) was found to play a significant role in non-fusion mediated cell death. Finally, the Nef protein (within the HIV Env) was analyzed for its ability to activate pDC and induce Type 1 IFNs. I observed that Nef is required for R3A-mediated induction of Type 1 IFNs, but this was specific to the cell type that produces the virus. Specifically, CD4 expression was intimately associated with Nef-mediated IFN induction. This study shed light on a possible mechanism that Nef plays to contribute to pathogenicity of HIV-1 Env.

Chapter 2: The Heptad Repeat (HR) 2 Domain Is A Major Determinant for Enhanced HIV-1 Fusion and Pathogenicity of A Highly Pathogenic HIV-1 Env.

Work previously performed by our group characterized HIV-1 viral isolates obtained from a rapid progressor in an attempt to glean pathogenic viral factors that may have contributed to the patient's alarming rate of disease progression. One isolate (denoted as

R3A) was found to cause significant CD4+ cell depletion in a relevant *ex-vivo* lymphoid model, compared to both a genetically similar isolate (R3B) and laboratory strain NL4.3. Further characterization showed that the V5-gp41 region of the Env significantly contributed to this difference in pathogenesis, either due to enhanced CXCR4 binding affinity or enhanced Env-mediated cell fusion ability.

To clarify this finding, I constructed Env chimeras to isolate the domain(s) within the V5-gp41 region that may contribute to enhance pathogenesis of R3A. The human thymus provides a prime host for HIV-1 infection and pathogenesis, and is representative of HIV-1 infection observed in other lymphoid organs. Using the HF-TOC system as an *ex vivo* infection model, I observed that the R3A gp41 ectodomain was both necessary and sufficient to cause CD4 T cell depletion, and this was due to enhanced fusogenicity. The enhanced rate of fusion was determined to occur for R3A Env due to the HR-2 domain, which also contributed significantly to the pathogenesis observed in HF-TOC infection. Although the NL4-R3B/A-Ecto and NL4-R3B/A-HR2 viruses caused significant depletion of CD4+ T cells compared to NL4-R3B, replication was not significantly enhanced. These data suggest that viral replication is not necessarily the prime determinant for pathogenesis. Interestingly, the various chimeric Envs were tested for sensitivity to AMD3100, and this demonstrated no correlation between fusion ability, CXCR4 binding affinity and downstream pathogenicity.

Further study focused on the HR-2 domain within the gp41 ectodomain, due to its prime involvement in enhanced fusion ability of R3A Env (as seen by both cell-cell and virus-cell fusion analysis). However, the pathogenic phenotype of the HR-2 chimera provided an intermediate level of depletion in the HF-TOC model, suggesting the involvement of the HR-1 domain in pathogenicity. Indeed, the fusion complex requires

complex interaction between coils that may be enhanced due to single mutations and interactions between the HR-1 and HR-2. Further analysis may dissect the role of the HR-1 (in concert with HR-2) in the pathogenic phenotype of the R3A gp41 ectodomain.

These findings clearly demonstrate that although viruses may show an enhancement of activities such as CD4-binding affinity and co-receptor affinity *in vitro*, enhanced ability of HIV virus to fuse to the host cell may be the most relevant determinant for direct cell killing. Enhanced fusion ability is correlated with syncytia-producing Envs, which are typically CXCR4-tropic and thought to evolve due to a lack of immune pressure. This may reflect upon R3A Env as a variant that emerged early in the patient R3 due to an absence of robust immune regulation. The enhanced level of pathogenesis observed with a highly fusogenic virus also lends fervor to the need to develop an assortment of efficacious fusion inhibitors, in the absence of a vaccine alternative.

Work previously described in Meissner et al showed that during NL-R3A infection of HF-TOC, addition of the fusion inhibitor C34 (at the height of viral replication) preferentially protected infected cells and impeded any further cell death. It is of note that those studies performed to examine the contribution of Env to R3A-pathogenesis all occurred using HIV Envs lacking functional Nef proteins. As it is well known that the Nef protein is a virulence factor due to several possible mechanisms, the Nef domain mutation was repaired by PCR strategies within the context of NL-R3A and R3B (unpublished work, Dr. L. Zhang). As expected, the inclusion of Nef protein expression produced a more robustly pathogenic version of R3A (within the NL4.3 background), characterized by enhanced immune activation as well as cell death even in the presence of C34. Interestingly, this same phenotype was not observed with NL-R3B, suggesting that another Env-specific domain may

contribute to the observed immune activation and uninfected cell death. My next effort was to use these Nef-containing R3 viruses to characterize both this immune activation as well as non-fusion mediated cell death using the HF-TOC system.

Chapter 3: The Pathogenic HIV-1 Env Activates pDC and Induces IFN to Contribute to T Cell Depletion in Human Lymphoid Organs.

In an effort to characterize the pathogenic phenotype and immune activation of (Nef containing) NL-R3A and R3B, *in vitro* experiments were performed using resting (as opposed to activated) PBMCs. After a 12 hour infection of PBMCs with virus, a Type 1 Interferon (IFN) Bioassay was initially performed to look at innate immune activation. Type 1 IFNs are known to be secreted by cells upon first interaction with various pathogens and provoke a large number of anti-viral responses. I observed that infection (at equivalent titers) with NL-R3A but not NL-R3B induced significant amounts of Type 1 IFNs, 12 hours after culture of cells with virus. To verify that the IFN bioassay was indeed Type 1 IFN specific, infection with NL-R3A was repeated in the presence or absence of IFNαβ-neutralizing antibodies. The specificity of this assay was validated in that IFN nAb significantly abrogated protection against an IFN-sensitive challenge virus. Using IFN nAb specific to IFNα or β, it was also determined that the bulk of protection was obtained by IFNα (data not shown). Both IFNα and IL-6 ELISAs were performed to quantify their presence as well as further characterize innate immune activation. In both assays, NL-R3A was found to induce significantly more of both proteins than NL-R3B, suggesting a substantially different level of innate immune activation that is Env-specific.

Recently, plasmacytoid dendritic cells (pDCs) have been shown by several groups to be the major IFN-producing cell type *in vivo* and respond to a host of pathogens such as HIV.

To test the function that pDCs may play in this innate immune activation phenotype, pDCs were either depleted or sorted and then cultured with HIV-1 for a short period of time. Bioassay analysis confirmed that resting PBMCs absent of pDCs did not induce detectable levels of Type 1 IFN (in contrast to total resting PBMCs). Purified pDCs demonstrated enhanced IFN induction due to NL-R3A over NL-R3B, in a dose-dependent fashion. From these data I conclude that NL-R3A is able to preferentially activate pDC, in an Env-specific fashion.

PDCs are known to express the CD4 receptor as well as both co-receptors CCR5 and CXCR4, so what level of interaction between HIV-1 and pDC is necessary for IFN induction? To answer this question, I cultured resting PBMCs with NL-R3A in the presence of several blocking drugs. Inhibiting CD4 binding (by the presence of either soluble CD4, antibody B12 or Leu3a) significantly inhibited IFNα induction. Although CD4 binding was found to be necessary, neither fusion (inhibited by C34) nor reverse transcription (inhibited by Nevirapine) was required for pDC activation. Interestingly, both Chloroquine and Bafilomycin A1 inhibited pDC activation. These drugs block various stages of endocytosis, suggesting that pDC must bind to HIV-1 via CD4, but then the cell must endocytose the virion to become activated.

Through various methods of *in vitro* analysis, Dr. Meissner previously demonstrated that the R3A V1V2 domain conveyed enhanced CD4 binding affinity to the R3A Env, compared to the R3B Env. Because CD4 binding was found to be necessary for HIV-1-activation of pDC, the V1V2 chimeras were utilized to confirm whether CD4 binding affinity might play a role in HIV-1-pDC activation. Upon infection of both resting PBMCs and purified pDC, the R3A V1/V2 domain conveyed the ability to induce IFN that was observed

with the parent R3A Env, while the R3B V1/V2 domain transferred the attenuated ability to induce IFN seen with R3B Env. From these data, I conclude that a highly pathogenic HIV-1 activates pDC (found in resting PBMC) through a CD4-dependent endocytotic pathway, and the Env V1/V2 domain that offers enhanced CD4-binding affinity mediates this activation.

A similar phenotype of differential Env-specific IFN induction was also observed after HIV-1 infection of human fetal thymus tissue. To determine the role that Type 1 IFN plays upon HIV-1 infection in the lymphoid environment, IFNαβ-neutralizing antibodies were added to thymic fragments prior to infection with NL-R3A. While the IFN nAb facilitated enhanced viral replication (data not shown), the depletion observed was not statistically different from non-drug treated infection. The addition of C34 (at the height of viral replication) protected cell depletion to a significant level, but the use of IFN nAb and C34 provided a synergistic level of protection in HF-TOC during infection by NL-R3A.

These data produce an interesting finding that begs the question: do Type 1 IFNs truly provide a pathogenic effect during infection of HIV-1? As I previously showed that the gp41 ectodomain of R3A provides for enhanced fusion-induced cell death as well as a V1/V2 domain that preferentially enhanced pDC activation, the V1/V2 chimera viruses were used to further dissect IFN-mediated cell death from fusion-mediated cell death in the HF-TOC system.

Upon infection of HF-TOC with NL-R3A, R3B and V1/V2 chimeras, enhanced IFN induction was observed by viruses carrying the R3A V1/V2 domains, 24 hours post infection. Introduction of the R3A V1/V2 domain into R3B enhanced both viral replication to levels similar to R3A, and CD4 depletion statistically different than parent NL-R3B. To

control for fusion ability as well as pDC activation ability, both C34 and IFN nAbs were added during the infection of HF-TOC with the V1/V2 chimeras.

In the case of HF-TOC infected with R3B/A-V1V2, viral replication was shown to be enhanced in the presence of IFN nabs. This suggested the R3A V1/V2 domains induced Type 1 IFNs that would suppress HIV-1 replication during infection. When CD4+ cell depletion was observed, it was found that addition of C34 alone did not protect cells from depletion while IFN nAb did. It is not surprising that C34 alone did not protect cells from depletion, as the virus carries the R3B gp41 ectodomain known for attenuated fusion ability. Hence, fusion is not a major player in this virus's depletion ability. Rather the R3A V1/V2 domain provides for enhanced pDC activation and IFN induction, which causes a more severe pathogenic phenotype (that is not fusion mediated). Interestedly, addition of both IFN nAbs and C34 again provided synergistic protection against the CD4+ depletion observed after infection of NL-R3B/A-V1V2.

Infection of HF-TOC with R3A/B-V1V2 also generated an interesting phenotype. Again it was observed that the presence of IFN nAbs increased viral replication, but it was further enhanced by the inhibition of fusion. When CD4+ cell depletion was observed, it was found that IFN nAb provided no protection against depletion, while addition of C34 did protect significantly. Unexpectedly, the addition of both IFN nAbs and C34 again synergistically protected cells against depletion, to an even more significant level than observed by infection with R3B/A-v1v2. The addition of C34 was expected to protect cells more robustly, because the virus carried the more highly fusogenic (and pathogenic) R3A gp41 ectodomain. It was quite interesting, however, to find such a significant synergistic protection observed by inclusion of both C34 and IFN nAb, as this virus carried the R3B

V1/V2 domain. Env carrying R3B V1/V2 domains were shown to induce only attenuated pDC activation, so the neutralization of IFN would not be expected to have such a robust effect in protection of cell death. These data provide insight into the concerted mechanisms that a pathogenic HIV-1 may use to deplete cells. The depletion of CD4+ cells occurs both through a fusion-induced pathway (observed by protection seen with C34) and also through an IFN-mediated pathway. This study clearly indicates the functional importance of both of the domains situated within the HIV-1 Env and their mechanisms of action used to induce both direct (fusion-mediated) and indirect (IFN-mediated) cell death.

For this story, many questions remain unanswered. On the side of the pathogen, I have observed that the V1/V2C2 domains play a significant role in pDC activation. As many amino acid changes exist within the V1, V2 and the C2 domains, further chimeras can be produced to test which domain plays the most significant role in pDC activation. This can also be used to specify mutations that contribute to CD4-binding affinity and preferential Env-induced pDC activation.

On the host side: how does IFN cause cell death? One group has recently suggested that TRAIL-mediated apoptosis is induced by pDC activation and IFN induction. Indeed, preliminary data in my hands also suggests a role for TRAIL, due to protection observed in the presence of an anti-TRAIL antibody during HF-TOC infection by NL-R3A (data not shown). However, several groups have shown the occurrence of Fas/FasL-directed apoptosis and necrosis as possible mediators of cell death as well. This analysis will be repeated as well as inhibition of other apoptotic pathways, to determine whether IFN-mediated cell death truly occurs through TRAIL.

Chapter 4: The HIV-1 Nef protein enhances Env to activate plasmacytoid DC through a CD4-dependent pathway.

The role that the Nef protein plays in the pathway of pDC activation and IFN induction was also questioned. As mentioned earlier, the pDC activation phenotype was only observed after the repair of Nef protein expression within the R3 Env. Specifically, infection of HF-TOC with NL-R3A (+ Nef) demonstrated significant depletion of CD4 cells in the presence of fusion inhibitor C34, in both percentage as well as total number of cells depletion. This indicated that a fusion-independent method of cell death was occurring, and this was Nef mediated. I previously found that Env played a role in differential pDC activation and bystander cell death (described above), so I questioned whether Nef also plays a role in this phenotype of innate immune activation. Using both NL-R3A and NL4.3 (+/- Nef) for infection of resting PBMCs, I conclude that IFN induction is regulated by the presence of Nef expression within the HIV Env. However, a complicated phenotype emerged when viruses were generated by A293T cell transfection as opposed to PBMC infection. Virus derived from PBMCs clearly demonstrated a Nef-dependent activation of pDCs, while A293T-derived viruses (at equivalent titer to PBMC-virus) did not significantly activate pDCs in the presence or absence of Nef. There are many differences between cell types that may factor into this phenotype, which may cause differential infectivity as well as surface protein expression on the virion. However, as one clear function of Nef protein relates to CD4-downregulation and HIV-1-Env mediated pDC activation is thought to be enhanced due to CD4-binding affinity, I questioned whether CD4 surface expression on the cell may factor into this observed phenotype of innate immune activation. Specifically,

roughly 60% of activated CD3+ PBMCs express CD4, while 293T cells are absent of CD4 expression.

Human CD4 surface expression can be induced on A293T cells upon transfection with a CD4-expressing construct. A293T cells +/- CD4 were transfected with HIV-1+/- Nef, to answer the question of CD4 function in viral production and innate immune activation. Viruses were harvested and used to infect resting PBMCs (at equivalent levels of p24/ml). Interestingly, IFN induction was again found to be Nef dependent, similar to the phenotype observed with PBMC-derived viruses. Also, I observed that IFN induction was CD4-dependent.

What we have now is a very interesting story that sheds light into a novel role for HIV-1 Nef in innate immune activation. These data suggest a model where HIV Nef protein utilizes the CD4 expression pathway to facilitate HIV Env expression on cell surface, which in turn provides for more HIV Env expression on the surface of virion after viral egress from the cell. When the HIV Env has enhanced CD4-binding affinity, this results in more robust virion-associated HIV Env binding and activation of pDC. To complete this story, several questions must be answered:

1) *Does CD4 downregulation by Nef enhance HIV Env expression on the surface of the virion?* This hypothesis can be examined by Western analysis for Env expression from both virus-producing cells and purified virions.
2) *What domain(s) of Nef are required for this CD4-dependent innate immune activation?* This question will be answered using various Nef mutants that carry mutations in the known functional domains of the Nef protein.

3) *How does the Nef protein contribute to pDC inactivation?* Clinical findings suggest that while HIV-1 initially activates pDC, re-occurring activation is attenuated if not inactivated. Our preliminary findings (using the Gen2.2 pDC cell line) suggest that HIV-1 infection of pDCs inactivated further stimulation by TLR9 ligand (CpG), and this inactivation was not observed upon infection with Nef-deleted virus. Further experiments will be performed to both validate this finding, as well as determine whether HIV-1 Env plays a role in this phenotype and whether other methods of activation are also affected by HIV/pDC interaction.

Conclusions:

Experimentation with a highly pathogenic HIV-1 derived from a rapid progressor has lent much insight into the nature of HIV-1 pathogenesis of the lymphoid microenvironment. This is particularly important as lymphatic and mucosal tissues present the major target for HIV-1 *in vivo*, and initial (acute) infection has been implicated as a major determinant for the severity of disease. HIV-1 infection and CD4+ cell depletion in the thymus has been shown to occur early in infection, and the substantial decrease in thymic emigrants has been shown to hasten AIDS progression.

Using the HF-TOC system as an *ex vivo* model for pathogenesis, aspects of the HIV-1 Env can be studied for their relative roles in cell death. From the total body of this presented work, I conclude that the R3A Env demonstrates enhanced pathogenic ability due to two major Env domains. The R3A gp41 ectodomain causes enhanced fusogenicity (primarily due to its HR-2 domain), which leads to fusion-induced death in both P24+ and P24- cells. Another (but equally important) domain, the V1V2 domain, presents a role in innate immune

activation (specifically seen with pDC) that causes bystander cells to die in a fusion independent method. In order for this to occur, the Nef protein also plays role that is presumably due to CD4-dependent enhancement of Env expression on the virion surface. This Env and Nef interaction has presented a surprising relationship to activate pDC, hence inducing a fusion-independent cell death on CD4+ lymphocytes. I propose a model where HIV-1 Nef protein is integral to CD4-downregulation of infected cells, such that resulting virion have enhanced HIV-1 Env surface expression. HIV-1 Envs that have demonstrated enhanced CD4-binding affinity (mediated by the V1V2 domain) are able to more efficiently activate pDC, and the resulting Type 1 IFN induction presents a fusion-independent pathogenic phenotype. Further activation of these pDCs may be inhibited due to these endocytosed HIV-1, by a yet unknown mechanism.

This body of work depicts the functions of a pathogenic HIV-1 to both readily fuse and activate pDC to cause CD4+ depletion, by both fusion-dependent and fusion-independent methods. These data suggest the need to develop both better fusion inhibitors as well as co-treatment with interferon-neutralizing therapeutics. In our model, inhibiting both pathways presents successful protection of CD4+ cells from HIV-1 depletion.