Evaluating Human Endogenous Retrovirus
and LINE-1 Retrotransposable Element
Antigens as Novel Targets for T cell Based
HIV-1 Vaccine Strategies

By
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A thesis submitted in conformity with the requirements for the degree of Doctor of
Philosophy, Graduate Department of Immunology, University of Toronto

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How does newness come into the world? How is it born? Of what fusions, translations, conjoinings is it made?

How does it survive, extreme and dangerous as it is? What compromises, what deals, what betrayals of its secret nature must it make to stave off the wrecking crew, the exterminating angel, the guillotine?


Evaluating Human Endogenous Retrovirus and LINE-1 Retrotransposable Element Antigens as Novel Targets for T Cell Based HIV-1 Vaccine Strategies, R. Bradley Jones, Doctor of Philosophy, Graduate Department of Immunology, University of Toronto, 2012.

Abstract.

The global HIV-1 pandemic is new only in the sense that it is the latest iteration in a conflict between humans and retroviruses that has spanned millions of years. Bearing witness to this, our genomes are littered with the DNA remnants of ancient retroviruses. These ‘human endogenous retroviruses’ (HERVs) are generally thought to be inert. In this thesis, I explore the hypothesis that HIV-1 has had to make a compromise in order to avoid extermination by a class of cellular defence factors that our cells evolved long ago in order to defend against ancient retroviruses. In disabling these defence factors to allow for its own replication, I posit, that HIV-1 enables the expression of the ancient retroviruses, as well as LINE-1 retroelements, in our genome. I propose to use this against the virus by targeted immune responses against HERV/LINE-1 antigens as a way of killing HIV-1-infected cells.
Acknowledgments.

Thank you to Douglas Nixon and Keith Garrison, for dreaming a dream with me, and to Mario Ostrowski and James Merson, for sharing in our vision and manifesting it into reality.

To Peter Loudon and the team at Pfizer Inc for being wonderful collaborators throughout this project.

To all of my students: Erick Duan, Jessica Wong, Kyryl Zagorovsky, Cole Stanley, Sara Holditch, Diana Hunter, Shariq Mujib, Vesna Mihajlovic, Eric Martin, Vivek John, Nabil Faruk, Nasra Aidarus and Blake Ziegler. Please take my gratitude with you into your bright futures.

To all of my friends and family. I apologize for all of the unanswered phone calls and unreturned messages. When I am in the grip of discovery the rest of the world disappears. Thank you for still being there when I came up for air.

To the faculty and students of the Department of Immunology. If I regret anything from my time here it’s not having taken the time to get to know you all better. I have always considered myself very lucky to be associated with such an amazing group of people.

To Jonah Sacha and Neil Sheppard, my brothers in science.

To my committee members – Tania and Alan. Thank you for your advice and support and for your confidence in me.

To the Ontario HIV Treatment Network for salary support throughout my PhD studies.

To the National Institutes of Health and the Canadian Institute of Health Research, for funding.

To my parents, for their unwavering support provided without either expectation or pressure.

To Randy, for being the foundation.
Lists of Abbreviations

Ad5 – Adenovirus serotype 5

BLCL – B cell lymphoblastoid cell line

CFSE – carboxyfluorescein diacetate succinimidyl ester

CTL – cytotoxic T lymphocyte

CML – chronic myeloid leukemia

CMV – cytomegalovirus

DC – dendritic cell

eGFP – enhanced green fluorescent protein

ELISPOT – Enzyme linked immunoSPOT

Env – Envelope (viral protein)

FBS – fetal bovine serum

Gag – Group-specific antigen (viral protein)

GFP – green fluorescent protein

GM-CSF – granulocyte macrophage colony stimulating factor

HERV – Human endogenous retrovirus
HIV-1 – Human Immunodeficiency Virus type 1

HML – Human MMTV-like

IFN-γ – Interferon gamma

JSRV – Jaagsiekte sheep retrovirus

LINE-1 or LINE-1 – Long interspersed nuclear element type 1

M-CSF – macrophage colony stimulating factor

MHC – Major histocompatibility complex

MMTV – Mouse mammary tumor virus

MuLV – murine leukemia virus

NHP – non-human primate

ORF – open reading frame

PBMC – peripheral blood mononuclear cells

PBS – primer binding site (in describing HERV nomenclature) OR phosphate buffered saline (elsewhere)

PCR – polymerase chain reaction

PD-1 – programmed death 1

qPCR – quantitative PCR
RPMI – Roswell park memorial institute 1640 medium

RT-PCR – reverse transcription polymerase chain reaction

SBBC – Sydney blood bank cohort

SFU – spot forming units

siRNA – small interfering RNA

TBP – TATA-box binding protein

TCR – T cell receptor

TGDA – Targeted genome difference analysis

TIM-3 – T cell immunoglobulin mucin domain containing molecule 3

TPRT – target-primed reverse transcription

UTR – untranslated region

VLP – virus like particle
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Manuscripts Arising from this Thesis:

Manuscripts for which material are directly incorporated into thesis:


**Jones RB, et al.** Comprehensive elimination of globally diverse HIV-1 primary isolate infected cells by HERV-K(HML-2)-specific CD8+ T cells. Under revision with *Immunity* – Chapter 4.

**Jones RB, et al.** HERV-K(HML-2) and LINE-1 specific T cell responses are detected at low frequency in HIV-1-infected subjects using 15mer peptides. Manuscript submitted to *Clin. Vaccine Immunol.* – Chapter 5.


Manuscripts related to HERV, LINE-1 or Tim-3 for which data are peripheral to thesis:


* Co-first authorship
Manuscripts unrelated to HERV and LINE-1 published over course of PhD training.


Thesis Overview.
Evaluating Human Endogenous Retrovirus and LINE-1 Retrotransposable Element Antigens as Novel Targets for T cell Based HIV-1 Vaccine Strategies, R. Bradley Jones, PhD, Department of Immunology, University of Toronto, 2011

Chapter 1:
This section provides a general overview of the current status of HIV-1 vaccine development efforts, highlighting the challenges posed by HIV-1 sequence diversity and summarizing strategies being explored to overcome this obstacle.

Chapter 2:
Detailed methods are provided for all of the experimental techniques employed in this thesis.

Chapter 3:
This section presents evidence for the de-repression of longinterspersed nuclear elements (LINE-1 or LINE-1) in HIV-1-infected cells. We observed the accumulation of high levels of LINE-1 DNA in HIV-1-infected primary CD4 T cells. Retrotransposition is presented as the likely etiology for this copy number increase, as is supported by the identification of novel LINE-1 genomic insertion sites in HIV-1 infected cells. The results from an in vitro retrotransposition assay further support the induction of LINE-1 retrotransposition in HIV-1-infected cells, and indicate that the HIV-1-Vif protein is necessary for this effect. As retrotransposition is dependent upon expression of both the
LINE-1 ORF1 and ORF2 proteins, this provides indirect evidence for their expression in HIV-1-infected cells. This is used as rational for studying the induction of LINE-1-specific T cell responses in HIV-1 infected subjects in the Annex to Chapter 4 and in Chapter 5.

**Chapter 4:**

This section presents rationale for targeting HERV-K(HML-2) antigens in HIV-1 vaccination strategies. We provide clear evidence for the rapid accumulation of spliced and unspliced HERV-K(HML-2) transcripts upon HIV-1 infection of primary CD4+ T cells. This is presented along with immunohistochemistry and western blot data supporting concomitant induction of HERV-K(HML-2) Gag and Env protein expression. We demonstrate comprehensive elimination of CD4+ T cells infected with diverse isolates of HIV-1, HIV-2 and SIV by a HERV-K(HML-2)-Env-specific T cell clone. This represents, to our knowledge, the first demonstration of specific killing of HIV-1-infected cells by a non-HIV-1-specific T cell. We also present data probing the mechanism of HERV-K(HML-2) induction in HIV-1-infected cells, including the requirement for the viral accessory protein Vif. The unprecedented breadth of reactivity of the HERV-K(HML-2)-Env-specific T cell clone highlighted in this chapter provides impetus for exploring the development of HERV-K(HML-2) targeted vaccines and immunotherapeutics.
Annex to Chapter 4:

Here we present preliminary data supporting that LINE-1 antigens may also provide effective surrogate markers for HIV-1-infected cells, by demonstrating that a LINE-1-ORF2p-specific CD4+ T cell clone specifically responds to autologous HIV-1-infected macrophages.

Chapter 5:

In this section we present the results of screening a diverse sampling of HIV-1-infected and uninfected subjects for HERV-K(HML-2) and LINE-1 specific T cell responses using pools of overlapping 15mer peptides. Responses to these antigens were observed only very infrequently, and efforts to detect them were hampered by false positive responses that were irreproducible with newly synthesized batches of peptide. The source of three of these false positive responses was traced to contamination with a single HIV-1-Gag derived peptide. This is presented as a caution for future studies aimed at detecting HERV-K(HML-2) and LINE-1-specific T cell responses in HIV-1-infected subjects. Two of the T cell responses presented in this chapter – one directed against HERV-K(HML-2)-Env and another directed against LINE-1-ORF2p are studied for their abilities to respond to HIV-1-infected cells in-depth in Chapters 4 and the Annex to Chapter 4, respectively.

Chapter 6:

Here we present the optimization, and early results of the implementation, of a dendritic cell based platform for the expansion of low frequency HERV-K(HML-2)-specific T cell
responses from PBMC. We demonstrate that, using this method, we are able to confirm the existence of responses previously detected by peptide ELISPOT, as well as to detect responses present below the level of detection of the ELISPOT assay. Two HERV-K(HML-2)-specific T cell lines, expanded in this manner, are shown to respond to CD4\(^+\) T cells expressing their cognate antigen, as well as to HIV-1-infected target cells. This provides early evidence that the anti-HIV-1 functionality of the HERV-K(HML-2)-specific T cell clone demonstrated in Chapter 4, is likely to be generalizable to other HERV-K(HML-2)-specific responses in other subjects. Plans for implementing this platform to assess a potential role for HERV-K(HML-2)-specific T cell responses in natural control of HIV-1 infection are also presented.

**Chapter 7:**

This chapter represents a departure from the primary focus of this dissertation. We have focused primarily on the goal of circumventing the issues of HIV-1 sequence diversity and mutability in the development of vaccines. Here we explore a means of overcoming another of the obstacles to the development of therapeutic vaccines - the progressive dysfunction of T cells, termed exhaustion, which is a hallmark of chronic viral infections. We demonstrate that expression of the inhibitory molecule Tim-3 is dramatically upregulated on both CD4\(^+\) and CD8\(^+\) T cells in chronic HIV-1 infection, and that expression levels correlate with clinical parameters of HIV-1 progression. We show that blocking the Tim-3 pathway, through the addition of either soluble Tim-3 or anti-Tim-3 antibody, improves the functionality of exhausted T cells. The generation of an effective
therapeutic HIV-1 vaccine may require a combination of strategies aimed at reversing T cell dysfunction and overcoming sequence mutability.

**Chapter 8:**

This results presented in Chapters 3 – 7 are summarized and discussed.

**Chapter 9:**

Proposed future directions for the work presented in this thesis are presented.
Chapter 1: Introduction

1.1 – Status of HIV-1 vaccine development efforts:

Despite more than 25 years of intensive research the development of a safe and effective HIV-1 vaccine remains elusive. An exploration of the factors underlying this protracted development is best framed in the context of what is known regarding the modes of action of successful vaccines for other pathogens.

1.1.1 – Successful vaccines for other pathogens and limitations in applying to HIV-1 vaccine development

The two pillars of successful vaccines have been, and continue to be, inactivation and attenuation, where the large majority of successful vaccines rely on inoculation with weakened or killed pathogens. A similar approach involving inoculation with related organisms which are less pathogenic than the target organism has also yielded success – most famously in the use of vaccinia virus to protect against variola virus (smallpox), a strategy which led to the eradication of smallpox in 1980. It is sobering to realize that the success of the large majority of licensed vaccines has emerged out of empirical observations and rarely been attributable to rational design based on knowledge of the immune system. Safety concerns precluding reliance upon these two pillars of vaccination in the development of an HIV-1 vaccine have forced a rational approach, exposing deficits in our understanding of the immune system, and provided impetus for an unprecedented level of study of the human immune system.
The use of attenuated or inactivated HIV-1 as a vaccine strategy was largely dismissed early in the epidemic due to safety concerns inherent in applying this method to a retrovirus. The replication of retroviruses, such as HIV-1, involves reverse transcription of the viral RNA genome into DNA and then integration of this provirus into the host genome. In this way, the viral genome becomes an inseparable component of infected cells and can persist, even in the absence of active viral replication, for the life of this cell. Should the infected cell happen to be a long-lived cell, such as a memory T cell, the host will retain the possibility of viral reactivation for many years. This is in contrast to viruses for which inactivated or attenuated vaccines have been successfully employed – such as poliovirus - which possesses a short-lived RNA genome. Compounding the risk involved in carrying persistent copies of proviral DNA, HIV-1 carries the potential to regain pathogenicity \textit{in vivo} via recombination between viral genomes and/or the acquisition of mutations. The latter case is of particular concern for HIV-1 which replicates with an error rate of $3.4 \times 10^{-5}$ mutations/bp/replication cycle nucleotides incorporated into nascent DNA (236).

Studies from the non-human primate (NHP) Indian rhesus macaque SIV infection model support the potential efficacy of attenuated HIV-1 as a vaccination strategy, while evidence from both this model and from observations of humans who accidentally became infected with attenuated HIV-1 validate safety concerns regarding this approach. In the Indian rhesus macaque SIV infection model live attenuated SIV vaccines have provided nearly complete protection against challenge with homologous virus (64, 134, 186, 202, 205, 219, 299). Vaccination with the attenuated virus SIVmac239Δnef has
further been shown to protect against acquisition upon repeated low-dose rectal challenge with the heterologous swarm isolate SIVsmE660, as well as to significantly reduce acute-phase viral loads of vaccinated animals upon acquisition (240, 241, 299). Unfortunately, these successes come with a very substantial downside, where SIVmac239Δnef animals not only failed to control chronic-phase viral replication but, in fact, displayed higher levels of viremia and more rapid progression than control animals. Bulk sequencing of circulating virus in these animals indicated extensive recombination between the vaccine and challenge strains, apparently resulting in virus that was more fit than either of the strains alone. Other macaques vaccinated with SIVmac239Δnef have progressed to AIDS in the absence of wild type virus challenge, and this has been associated with the acquisition of point mutations which restored expression of a truncated form of Nef (122, 259). In humans, the risks of vaccination with attenuated HIV-1 viruses are illustrated by a unique case where 8 individuals were inadvertently infected with an attenuated strain of HIV-1, containing gross deletions in the nef/long terminal repeat region of the viral genome, by receiving blood transfusions from a single donor (28, 70, 164). Despite this single source of infection, subjects in this Sydney blood bank cohort (SBBC) have displayed heterogeneous clinical outcomes – with 3 subjects exhibiting ongoing ‘elite control’, defined by stable CD4 counts and undetectable viral loads, and 2 subjects eventually experiencing declines in their CD4 counts after many years of control (the other 3 subjects died of causes unrelated to HIV-1 and thus cannot be classified into one of these two groups). The eventual progression of some subjects in the SBBC has been associated with a progressive loss in residual nef sequence towards the minimal sequence
elements required for HIV-1 replication (53). Together, these data from both humans infected with attenuated HIV-1, and rhesus macaques infected with attenuated SIV provide a small sampling of the diversity of mechanisms by which attenuated lentiviruses can regain pathogenicity. Furthermore, these data illustrate considerable heterogeneity in clinical outcomes for subjects infected with attenuated lentiviruses, comprising a substantial safety concern for any attenuated HIV-1 vaccine.

1.1.2 – Antibody based approaches to HIV-1 vaccine design

In lieu of relying upon attenuation or inactivation for establishing protective immunity, the HIV-1 vaccine field has been forced to attempt to replicate protective aspects of the immune response using some form of a synthetic component of the HIV-1 virus, or a mimetic thereof. The protection afforded by most existing vaccines is attributable primarily to antibody responses, as is supported by several lines of evidence - most conclusively by the demonstration that passive transfer of vaccine-induced antibodies engenders protection from a host of pathogens including measles, rabies and smallpox (1, 93, 100, 114, 144). Strategies aimed at specifically eliciting antibody responses by inoculating with recombinant pathogen-derived proteins were therefore developed, and proved successful for vaccines targeting the Hepatitis B surface antigen, Lyme outer surface protein A, and others (55, 86, 88, 197, 235). The first HIV-1 vaccine efficacy study therefore explored this strategy, by attempting to elicit protective antibody responses by vaccinating volunteers with recombinant HIV-1-Env gp120. Although this approach succeeded in eliciting antibody responses in the majority of volunteers, these antibodies proved incapable of neutralizing primary isolates of HIV-1 and provided no
protection from either acquisition or progression (55, 88, 235). Much has been learned from this failure, and from subsequent studies, and it is now generally accepted that antibodies which target functionally relevant and conserved epitopes on intact trimeric HIV-1-Env will be required to afford antibody-based protection from HIV-1 (159, 174, 256, 260, 300). The discovery of a number of monoclonal antibodies, isolated from infected subjects, that are capable of broadly neutralizing diverse isolates of HIV-1, provides hope that such responses could be elicited by vaccination. A number of significant technical challenges must be overcome in developing an immunogen capable of engendering antibody-based protection from HIV-1 and this remains an area of intense focus in the HIV-1 vaccine field.

1.1.3 – T cell based approaches to HIV-1 vaccine design

With the current lack of immunogens capable of eliciting broadly neutralizing HIV-1-specific antibody responses, considerable efforts have been directed at developing a vaccine that provides protection by generating T cell immune responses – also known as cellular immunity – directed against the virus.

1.1.3.1 – Biology of T cells

T cells are a type of lymphocyte that develop in the thymus into one of two primary lineages, distinguishable by the expression of either CD8 or CD4 proteins on the cell surface. In contrast to antibodies, which target extracellular antigens, T cells respond to antigens present within cells – either produced by the cell itself, or taken up by the cell and processed internally. A specialized set of protein molecules called MHC-I and MHC-
II provide T cells with a type of window into the contents of a target cell by binding to fragments of cellular proteins (peptides) and carrying these to the cell surface where they can be presented to T cells. Recognition of these peptides, also referred to as ‘T cell determinants’ or ‘T cell epitopes’, thus occurs in the context of MHC-I or MHC-II and is manifested by the T cell receptor (TCR).

The TCR is a member of the immunoglobulin super-family of proteins that is uniquely expressed on T cells. Generally speaking, each T cell has one specific form of TCR, however due to incomplete allelic exclusion of either the α or β chains of the TCR, multi-specific T cells have been reported (34, 182, 227). The TCR is exquisitely sensitive at detecting a corresponding T cell determinant in the context of either an MHC-I or an MHC-II molecule. The repertoire of TCRs, and hence of T cell specificities, in any given individual is vast - to the point that for any given antigen that an individual could potentially ever be exposed to there exists a pool of T cells circulating in the body with the ability to specifically respond to that antigen. Antigen-specific T cells thus exist in an inactive ‘naïve’ form in the body prior to any exposure to that antigen. In other words, an individual who has never been exposed to HIV-1 in any form has, in their body, a pool of T cells with the ability to specifically recognize HIV-1 derived peptides, as would a person living in the 1900’s before HIV-1 had ever infected a single human. The incredible diversity of TCR specificities is generated by a random process of recombination of germ-line encoded DNA elements, followed by mutation – with the entire process then directed by positive selection for successful forms of the TCR and
negative selection for unsuccessful or undesirable forms (such as those with the potential to react with peptides derived from self proteins) – see (129) for review.

The CD4+ and CD8+ subsets of T cells, mentioned above, recognize antigens derived from distinct sources and respond with different effector functions. CD4 and CD8 are both immunoglobulin super-family molecules which, when expressed on the surface of a T cell, act as co-receptors to the T cell receptor which are indispensable for the activation of T cell effector functions. CD4 binds to MHC-II and thus acts as a co-receptor for T cells which recognize peptide presented in the context of MHC-II, while CD8 binds to MHC-I and acts as a co-receptor for T cells which recognize peptide presented by MHC-I. The differential functions of CD8+ and CD4+ T cells are thus tied to differences in the sources of antigens presented by MHC-I versus MHC-II. MHC-I is loaded, in the endoplasmic reticulum, with peptide antigens derived from proteasomal degradation of intracellular proteins, including proteins produced by the cell. In contrast, MHC-II is loaded in acidified endosomes, and thus primarily presents peptides derived from extracellular antigens. The presentation of a viral antigen by MHC-I on a cell therefore generally indicates that this cell is itself producing viral antigens and is infected, whereas the presentation of a viral antigen by MHC-II on a cell does not directly implicate infection of that specific cell, but rather indicates the presence of virus in the surrounding environment. The predominant effector responses of T cells is appropriate to these scenarios, with CD8+ T cells responding to infected cells presenting viral antigens on MHC-I by killing the target cells, and CD4+ T cells responding to recognition of viral antigens on MHC-II predominately by providing signals which further activate B cells
and CD8\(^+\) T cells in the environment. These differing roles for CD8\(^+\) and CD4\(^+\) T cells have earned them the labels: killer and helper T cells, respectively. This dichotomy is not absolute, as CD4\(^+\) T cells with direct cytotoxic activities have been described, and CD8\(^+\) T cells can express signaling molecules and secrete cytokines capable of providing ‘help’, however the generalization is more useful than it is misleading. It should also be noted that both CD8\(^+\) and CD4\(^+\) T cells respond to antigen by producing cytokines, such as IFN-\(\gamma\) which induces an antiviral state in proximal cells, and by producing chemokines such as MIP-1\(\beta\) which serve to attract additional effector cells to the site of inflammation and, in the case of MIP-1\(\beta\), to compete with the HIV-1-Env protein for binding to the CCR5 receptor.

1.1.3.2 – Evidence that T cell responses contribute to natural control of HIV-1 infection

The interest in the HIV-1 vaccine field in harnessing cellular immunity as a vaccination strategy has focused largely around CD8\(^+\) ‘killer’ T cells, also known as cytotoxic lymphocytes (CTL). The bias in studying CD8\(^+\) versus CD4\(^+\) T cells is primarily due to the more direct mechanism of action of the former and the lack of interdependence with antibody responses, given their known limitations against HIV-1. It should also be noted, however, that the preferential study of CD8\(^+\) T cells is also owed to the development of MHC-I tetramer technologies which allow for direct enumeration of antigen-specific CD8\(^+\) T cells by flow cytometry. The development of MHC-II tetramer technologies has lagged substantially behind due, in part, to the lesser stability of MHC-II complexes. The rational for moving forwards with CD8\(^+\) T cell based HIV-1 vaccination strategies is based on five primary lines of evidence suggesting that CD8\(^+\) T cells may
play an important role in natural control of HIV-1 infection. Firstly, the emergence of HIV-1-specific CD8$^+$ T cell responses following infection is temporally associated with a $10^2 - 10^3$ fold reduction in HIV-1 viremia during acute/early HIV-1 infection. Such is not the case for the emergence of HIV-1-specific antibodies which appear only several weeks after viremia has declined. Secondly, in the rhesus macaque SIV infection model the depletion of CD8$^+$ T cells has been shown to lead to a dramatic increase in viral load and rapid progression (261). Thirdly, in infected humans it has been established that there are strong linkages between the possession of certain MHC-I alleles and clinical progression, with over-representation of HLA-B57 and B27 alleles in subjects who control viremia and, conversely, over-representation of HLA-B35-Px (B*3502/3503/3504/3501) in subjects who progress rapidly to AIDS. Given that a primary function of MHC-I alleles is the presentation of virus-derived peptides to CD8$^+$ T cells these observations comprise strong evidence of an important role for these cells in natural control of HIV-1 infection. An analogous situation exists in the rhesus macaque SIV infection model where the Mamu-B*08 and Mamu-B*17 alleles are over-represented in animals which exhibit natural control of infection. The infection of Mamu-B*08$^+$ animals with SIV engineered to contain point mutations in immunodominant epitopes resulted in impairment of viral control as compared to animals infected with wild-type SIV (282). This provides clear evidence that CD8$^+$ T cells are the important effectors in Mamu-B*08-mediated control. Fourthly, CD8$^+$ T cells are known to exert sufficient pressure on targeted epitopes to drive the emergence of escape mutations. These mutations sometimes come at a substantial fitness cost to the virus, as evidenced by rapid reversion of the virus to wild-
type upon transmission to a subject lacking the MHC-I allele needed to present this peptide to T cells (the restricting allele) (5, 91, 169). Finally, CD8$^+$ T cells isolated from HIV-1-infected subjects show a clear ability to eliminate infected cells and suppress viral replication in vitro (304).

1.1.3.3. Rationale for T cell based HIV-1 vaccines

Owing to their mode of action, T cells can only respond to HIV-1 once it has infected a target cell. This is in contrast to antibody which can bind to extracellular virus and prevent it from ever establishing an infection. Given this limitation, it is generally accepted that a T cell based HIV-1 vaccine is unlikely to succeed in provided sterilizing immunity – that is, to prevent a vaccinee from ever acquiring infection. Rather, the goal of T cell vaccination is to enable a host to better suppress viral replication both in the acute and chronic phases of infection (Fig. 1.1).
Fig. 1.1. Proposed effect on HIV-1 viral replication of a hypothetical T cell based vaccine. Adapted from ‘Towards an AIDS vaccine’(288). The red line depicts HIV-1 viral load over the course of a typical natural infection. The green line, tracing lower acute-phase and set-point viral loads, depicts the proposed course of infection in an individual immunized with a hypothetical T cell based HIV-1 vaccine.

The benefits to the host of enhanced immunological control of replication are apparent – a reduced burst of viral replication during the acute phase of infection would result in a lesser degree of depletion of memory CD4\(^+\) T cells (39), while a reduced chronic-phase viral load set-point would be predicted to delay or prevent progression to AIDS. It is important to note, however, that since the risk of transmitting HIV-1 is correlated with viral load, and markedly reduced at levels below 1,700 copies of HIV-1 RNA/ml of blood plasma, the suppression of acute and set-point viral load levels by a successful T cell
based vaccine would also be predicted to prevent transmission of HIV-1 at a population level (110). Thus, while the development of a vaccine that elicits both protective antibody and T cell responses would be ideal, in the absence of a means of eliciting a protective antibody response, an effective T cell based vaccine would represent a viable means of curbing the HIV-1 pandemic.

1.1.3.3. Development of T cell based vaccination strategies in non-human primate SIV infection models

In the absence of a small animal model for HIV-1 infection, the challenge of non-human primates (NHP) with simian immunodeficiency virus (SIV) has emerged as the standard model for HIV-1 vaccine development. This model recapitulates many of the immunopathological features of HIV-1 infection including the infection and depletion of activated CD4+ T cells, leading to the acquisition of opportunistic infections and death (6, 7, 147, 171, 195, 226, 287). In evaluating the applicability of NHP challenge studies to HIV-1 vaccine development it is important to distinguish between homologous versus heterologous challenge. In a homologous challenge, the sequence of the challenge virus is identical to that of the viral antigens incorporated into the vaccine. This effectively negates the critical issue of viral sequence diversity and, while useful in testing some vaccine concepts such as the intrinsic ability of SIV-specific CD8+ T cells to suppress viral replication, does not simulate the exposure to diverse HIV-1 viruses that human vaccines would encounter in natural exposure. Heterologous challenges represent a more realistic test of vaccine efficacy, where the sequence of the SIV challenge virus is mismatched from the vaccine SIV sequences. In a more stringent form of heterologous
challenge the viral inoculum comprises, not a single clone of virus, but a heterogeneous swarm of viruses – whereby vaccine-induced immune responses must be reactive against a variety of viral sequences in order to provide effective protection.

It has become very clear, from a number of studies, that T cell based vaccines can protect Indian rhesus macaques from homologous challenges with SIV. This was first demonstrated using a DNA prime, recombinant adenovirus serotype 5 (rAd5) boost vaccination strategy incorporating SIV Gag, Tat, Nef, and Rev antigens (296). Env was omitted from this vaccine approach to preclude the elicitation of neutralizing antibody responses such that any observed effect could be attributed to T cell responses. While this vaccine did not protect from acquisition of SIV infection, it succeeded in reducing peak viremia from a geometric mean of 5x10⁷ copies/ml of viral RNA to 4x10⁶ copies/ml and chronic-phase set-point viremia from 1.49x10⁵ copies/ml to 5.3 x 10³ copies/ml. In a second study it was demonstrated that a rAd26 prime followed by a rAd5 boost vaccine regimen reduced peak and chronic-phase viremia by similar degrees and moreover that this control was durable to more than 500 days post-infection. Thus, if vaccine and challenge SIV sequences are matched, cellular immune responses are capable of achieving the goals of T cell based vaccination. It is less clear, at the moment, whether T cell based vaccines are capable of protecting Indian rhesus macaques from challenge with heterologous virus. Although some studies have provided encouraging results, it has become apparent that the challenge viruses used in these – the swarm SIVsmE660 – does not provide a very stringent test of protective immunity as many non-vaccinated control animals go on to control chronic-phase viremia (295). Thus the cumulative evidence from
stringent non-human primate models supports that an effective T cell based vaccine against HIV-1 could be developed were it not for the critical factor of sequence diversity.

1.2 – HIV-1 variability and mutability as obstacles to vaccine development.

HIV-1 sequence diversity manifests as a challenge to vaccine development on two fronts. Firstly, variation in circulating HIV-1 strains ensures that no virus that a vaccinee could be exposed to in the field will exactly match the sequence of the vaccine antigen. In the case that the virus differs from the vaccine sequence at an epitope targeted by either antibody or T cells, this may render the corresponding immune response completely ineffective. Secondly, the relentless viral mutation and evolution that occurs within an infected subject poses a serious challenge to accomplishing the T cell vaccine goal of enduring control of viral replication.

1.2.1 – Global diversity of HIV-1.

Phylogenetic analyses support the idea that three independent cross-species transmission events gave rise to the three groups of HIV-1: M, O, and N. Group M dominates the global HIV-1 pandemic, and is thought to have been transmitted from chimpanzees between 1915 and 1941 (154). This group has diversified rapidly, and at present is classified into the subtypes and sub-subtypes A1, A2, A3, A4, B, C, D, F1, F2, G, H, J and K (277). Far from being an end-stage of viral evolution, this represents only a snapshot in an ongoing dynamic process moving towards even greater variability. The
rapid evolution of HIV-1 is seeded by the infidelity of its reverse transcriptase (RT) enzyme which incorporates an estimated one error per $3.4 \times 10^{-5}$ mutations/base-pair/replication-cycle into the $\sim 10^4$ base viral genome (231). Given that upwards of $10^{10}$ virions can be produced within a single individual in one day, the variety of evolution paths being sampled globally by HIV-1 is staggering. The high replication rate and rapid turn-over of HIV-1 virions accounts, in part, for the tremendous diversity of HIV-1 in comparison even to other retroviruses which undergo reverse transcriptase steps, such as HTLV-I. However, it has also been proposed that strong selective pressures being exerted upon HIV-1, and a tolerance for change, may also be contributing factors (153).

The challenge posed to vaccine development by this diversity can be put into perspective by considering that a $<2\%$ variation in the amino acid sequence of influenza hemagglutinin can result in the loss of cross-reactivity of the polyclonal antibody response to the influenza vaccine and require a change in the vaccine strain. By comparison, the amino acid sequences of HIV-1-Env can differ by as much 20% within a single subtype, and by more than 35% between subtypes (97). In the absence of an immunogen that is capable of eliciting antibody responses of extra-ordinarily broad reactivity, the prospects for developing a single, globally effective, antibody-based HIV-1 vaccine are grim.

Due to their mode of action, T cells are not limited to targeting the variable HIV-1-Env protein – but rather can be directed against more conserved viral proteins that are expressed intracellularly. The HIV-1-Gag and Pol proteins would appear to represent particularly promising targets, with regions of both proteins varying by as little as 8%
between clades. Indeed cross-clade reactivity of CD8$^+$ T cells has been frequently reported, either by the detection of ex vivo CD8$^+$ T cell responses using synthetic peptides corresponding to mismatched subtypes (ex. screening a subject infected with subtype C virus with synthetic peptides corresponding to subtype B sequence), or by directly testing HIV-1-specific CD8$^+$ T cell clones for recognition of synthetic peptides representing diverse subtypes (9, 22, 44, 46, 47, 60, 65, 77, 85, 89, 92, 98, 99, 142, 178, 180, 230, 249, 297, 308). This has lead to the suggestion that the breadth of HIV-1-specific T cell responses may allow for the development of a universal vaccine against diverse subtypes.

A more recent study, however, has demonstrated that cross-reactivity of HIV-1-specific CD8$^+$ T cells with cells pulsed with exogenous synthetic peptides does not necessarily reflect cross-clade anti-viral activity (25). The basis for this discrepancy is likely that the functional avidity of CD8$^+$ T cells for variant T cell determinants from non-autologous clades is generally less than for autologous clades. Thus, while these T cells may cross-react with supra-physiological levels of exogenous peptide, they are unable to effectively recognize lower levels of cell-surface T cell determinants derived from endogenous infection. These findings support the idea that the cross-clade reactivity of HIV-1-specific T cells has been over-estimated, and that efforts to develop a T cell based vaccine that could be universally applied to diverse clades may have been misguided. Notably lacking from the literature at present are studies which directly assesses the breadth of cross-clade reactivity of HIV-1-specific CD8$^+$ T cells in a more physiologically relevant in vitro viral suppression assay. In the absence of such studies it is difficult to evaluate the extent to which the global diversity of HIV-1 will compromise the efficacy of vaccine-induced T
cell responses – however it is clear that antigenic variation does provide a considerable barrier to vaccine development.

1.2.2 – Population-level HIV-1 diversity.

While the development of a universal HIV-1 vaccine that could be used globally against diverse subtypes represents the ideal, in reality even the invention of a vaccine with a reasonable degree of efficacy against a single subtype would represent a tremendous breakthrough. In considering the impact of HIV-1 sequence diversity on the development of a region-specific HIV-1 vaccine it is important to consider the interactions of the virus with the local population and the pressures that this would exert on viral evolution. As the MHC-locus is the most polymorphic region of the human genome, and the MHC-I alleles encoded in this region exert direct immunological pressure on HIV-1 sequences through CD8$^+$ T cell responses, it is not surprising that HIV-1 has been observed to adapt to avoid MHC-I-restricted immune responses at a population level (207). Thus, although Gag antigen sequences may only vary by 5-10% within a subtype, these mutations do not occur at random, but are focused on amino acid residues that allows for HIV-1 to escape from CD8$^+$ T cell responses that are restricted by common MHC-I alleles. The fixation and transmission of stable T cell escape mutations by HIV-1 has been directly observed in cases of vertical transmission of HIV-1 from mother to infant, where escape mutations to an HLA-B27 restricted T cell determinant sequence in Gag were selected in the mother, transmitted to the infant, and then maintained regardless of whether the infant was HLA-B27$^+$ (109). It has further been inferred, from the observation that HIV-1 T cell determinants are clustered in conserved
regions of the viral proteome, that potential T cell determinants have been selectively erased from regions of the virus where such changes can be tolerated (309). Of note, however, the capacity for HIV-1 to escape from CD8$^+$ T cell responses at the population level is not without limitations. The acquisition of escape mutations within certain T cell determinants, such as the HLA-B57/5801 restricted TSTLQEQLIAW, comes at a clear fitness cost to the virus. This is evidenced by the observation that when viral variants with escape mutations in this sequence are transmitted to HLA-B57/5801$^-$ hosts (unable to present the determinant to T cells), the sequence reverts back to wild-type (169). In a similar vein, it has been observed that the transmission of viruses with escape mutations in HIV-1-Gag to subjects matched on the restricting HLA-allele is associated with reduced viral load in recipients – attesting to an ongoing fitness cost to the virus in the face of sustained immunological pressure (104). Thus in the ongoing co-evolution of HIV-1 within populations, the continued accumulation of escape mutations is not inevitable, but rather is balanced and limited by purifying selection.

The complex evolutionary dynamic between HIV-1 and a host population must be considered in developing T cell based vaccines. A vaccine which elicits responses to determinants with low fitness barriers for immune escape is likely to simply drive fixation of escape mutations at the population level. A vaccine, however, which elicits responses to determinants with high barriers to escape (such as highly functional regions) may force the virus circulating in the population to a reduced fitness level. In evaluating the outcome of the only T cell based HIV-1 vaccine to have undergone a phase IIB clinical trial, the Merck STEP trial, it would appear that the vaccine-induced T cell
responses fell into the former category (discussed below). Overall, the state of the art points to exogenous HIV-1 sequence diversity (the variability of sequences to which an individual could be exposed) comprising a significant, but not insurmountable, barrier to vaccine development. It is reasonable to suggest that a vaccine could be developed that would elicit broad T cell responses against multiple conserved determinants and that such a vaccine could be successful in targeting the majority of circulating HIV-1 strains, at least at a regional level.

1.2.3 - HIV-1 diversification within an infected host.

By far the greater barrier to the successful implementation of T cell based vaccines against HIV-1 is the diversification of the virus within an infected host. The stated goal of a conceptual T cell based HIV-1 vaccine is not to prevent infection, but to elicit protective immune responses capable of exerting enduring suppression of viral load. Given the incredible propensity of HIV-1 to mutate to avoid selective pressures, it is difficult to conceive of how, in the context of ongoing viral replication (albeit low level), the virus would fail to eventually acquire escape mutations for vaccine-elicited T cell responses in at least a subset of vaccinees. This is supported by studies of rhesus macaques that were vaccinated with env/gag plasmid DNA and then challenged with the pathogenic SHIV-89.6P virus, where early vaccine-mediated immune control of virus replication was lost in association with the acquisition of escape mutations (21). In one particularly ominous case, the mutation of a single amino acid in an immunodominant epitope was associated with an abrupt increase in viral replication leading to death from AIDS-related complications.
Insights into how we may expect viral diversification to impact upon vaccine-mediated immunological control in HIV-1-infected humans can be gained from studying diversification in natural HIV-1 infection. Recent studies have demonstrated that in ~75% of sexual transmissions of HIV-1, infection is established by viruses of a single lineage (2, 108, 112, 143, 166, 253). The viral population remains quite homogenous at peak viremia and then begins to diversify concomitant with the emergence of HIV-1-specific CD8+ T cell responses and the corresponding reduction in HIV-1 viral load (8, 48, 106, 221). The rates of acquisition of escape mutations in CD8+ T cell determinants during chronic progressive HIV-1 infection are much more limited, and these mutations appear to have only a modest impact on viral load (139, 150). However, this is likely due more to the apparent ineffectiveness of CD8+ T cell responses in this context than it is reflective of an inability of the virus to further diversify. The capacity for HIV-1 to mutate to escape effective vaccine-induced T cell responses is most likely best reflected by the rapid mutation observed during the effective immunological pressure exerted during acute HIV-1 infection. The most direct evidence for the ability of HIV-1 to mutate to escape vaccine-elicited CD8+ T cell responses is that emerging from the only T cell based vaccine to have undergone a phase IIB clinical trial – the Merck STEP trial.

1.2.4. Sequence diversity underlying the failure of the Merck STEP HIV-1 vaccine trial.

Recently, the T cell based HIV-1 vaccine concept was tested, by the pharmaceutical company Merck. In the phase IIB ‘STEP’ trial. The study protocol involved randomizing 3000 HIV-1 seronegative volunteers to receive three injections of
either a recombinant adenovirus vector (MRKAd5) encoding HIV-1 gag/pol/ nef or a placebo (42). This vaccine platform was selected based on its ability to elicit potent T cell responses, and HIV-1 env was purposely excluded from the regimen to negate any role of neutralizing antibody responses (49, 238). Vaccination with recombinant Ad5 induced T cell responses in 75% of vaccinees. These responses were modest in magnitude (163-686 spot-forming cells/10^6 PBMC by IFN-γ ELISPOT) and, targeted a limited breadth of T cell determinants (average of one determinant each from Gag, Pol and Nef) (198). The STEP trial was halted at the first interim analysis because it met the prespecified futility boundaries. Vaccinated subjects were neither protected from acquisition of HIV-1, nor displayed any improvement in levels of acute phase or set-point viral loads. A leading hypothesis for the lack of efficacy of this vaccine is that the vaccine-elicited HIV-1-specific CD8^+ T cells did not have sufficient breadth to recognize epitopes in transmitting viral strains. Alternately, rapid evolution of viruses which established a foothold in these subjects could have driven the emergence of escape mutations before infection could be cleared. These theories are supported by a recent study which provides evidence that founder viruses in vaccinees were more divergent from the vaccine sequence in potentially targeted T cell determinants than those from placebo recipients (244). These results are driving an emerging consensus in the field that a successful T cell based vaccine will have to incorporate innovative strategies to deal with the sequence diversity and mutability of HIV-1.
1.3 – Vaccination strategies being explored to overcome HIV-1 variability.

The accumulation of a vast amount of HIV-1 sequence information over the past 30 years, coupled with a wealth of knowledge regarding the HIV-1 T cell determinants targeted by the human immune system, has allowed for the emergence of a number of strategies aimed at increasing the likelihood that a vaccine-elicited T cell response will recognize the sequence of transmitted viruses.

1.3.1 – Polyepitope vaccines.

The premise behind the polyepitope vaccine strategy is that, in a natural HIV-1 antigen, some T cell determinants represent more attractive targets than others. Thus, rather than immunizing with a natural protein and allowing the immune system to select which determinants to target, the polyepitope approach allows for investigators to select very specific determinants on which to focus the immune response. This would be achieved, in theory, by stringing these determinants together into an artificial vaccine antigen. The selection of highly conserved determinants, in particular those known to exact a fitness cost in exchange for immune escape, would present an elegant means of mitigating the effects of HIV-1 variability. Unfortunately, although the polyepitope strategy has proven immunogenic in mice and in rhesus macaques, two separate trials in humans have yielded dismal immunogenicity. The mechanisms underlying this outcome are not presently understood. It is possible, however, that these setbacks represent a
surmountable technical hurdle, and that the general approach may yet prove valuable (107, 113, 130, 176, 215, 223, 292, 293).

1.3.2 – Targeting conserved regions of HIV-1.

The failure of the highly artificial polyepitope immunogens to elicit CD8⁺ T cell responses, despite efforts to optimize epitope processing, exposes gaps in our knowledge of how the larger context of T cell determinants in proteins influences their immunogenicity. This has led to the consideration of a strategy that represents something of a compromise between vaccinating with natural proteins and vaccinating with strings of isolated T cell determinants. This approach comprises linking together longer sections of the most conserved regions of HIV-1 proteins into chimeric immunogens. This concept has been tested in the mouse using an immunogen which incorporated the 14 most conserved regions of the HIV-1 proteome (170). Both BALB/c and HLA-A*0201 transgenic mice generated T cell responses to this vaccine. Based on these results a vaccine antigen is being moved into small human safety and immunogenicity trials.

1.3.3 - Central sequence vaccines.

In a third approach, vaccination is performed using full-length HIV-1 proteins that have been designed to be phylogenetically ‘central’ to the circulating HIV-1 strains that the vaccine is targeting. This can be performed on a regional level or, more ambitiously, on a global level. There are three related approaches to defining a central sequence – all of which aim to minimize the genetic distance between the designed immunogen and a given viral isolate that a vaccinee could be exposed to. The first strategy is to reconstruct an ancestral sequence which represents a hypothetical precursor at an ancestral node in a
phylogenetic tree – for example near the base of a given clade, or at the base of group M (76, 97, 157). The second is to construct a consensus sequence simply by determining the most common amino acid at every position in the protein and generating a synthetic construct representing this sequence (95, 97). The third approach aims to generate a ‘center-of-tree’ sequence which minimizes the sum of the distances to all branch tips in a phylogenetic tree representing the target group of HIV-1 strains (218, 243). In general, centralized antigens reduce the genetic distance between a vaccine protein and that of a circulating isolate by about half – as compared to a natural HIV-1 antigen (97).

The benefit of reducing sequence mismatches between vaccine and exposure virus sequences may be balanced by drawbacks – however these are primarily hypothetical in nature at the present time. Proteins designed from consensus sequences and center-of-tree sequences represent compromises between different sequences and don’t actually match with any known or predicted HIV-1 sequence. This raises the possibility that these proteins may not represent actual functional proteins and may not fold properly. Ancestral sequences are somewhat further removed from this possibility as they, at least in theory, represent the sequence of an actual precursor virus. Although these concerns cannot be completely dismissed, it is most encouraging to note that all consensus, ancestral and ‘center-of-tree’ HIV-1 sequences that have been tested to date are expressed well and are immunogenic (95, 97, 156, 157, 291). Another potential drawback to immunizing with ancestral HIV-1 sequences, which doesn’t appear to have been picked up by the field, is tied to the hypothesis that HIV-1 progressively evolves to accumulate stable escape mutations in T cell determinants in regions of the viral proteome that can
accommodate such changes (discussed above in 1.2.1). If this hypothesis is correct, and the divergence of HIV-1 group M has been driven in part by T cell mediated immune pressure, then an ancestral HIV-1 sequence would be riddled with immunogenic T cell determinants with low barriers to immune escape. Although this is particularly apparent for ancestral sequences, it also represents a hypothetical flaw for consensus and center-of-tree sequences as both will fall closer to the node on a phylogenetic tree (both more similar to ancestral sequence than the average natural sequence). Ironically, therefore, these strategies aimed at overcoming HIV-1 sequence diversity by engineering a central sequence may in fact exacerbate the problem – at least in the context of a T cell based vaccine where enduring control of low-level viral replication is more of a challenge than recognition of transmitted viruses.

1.3.4 – Mosaic vaccines and polyvalent mosaic vaccines

In this approach, computational algorithms are applied to HIV-1 sequence databases to generate artificial HIV-1 antigen sequences which maximize coverage of potential T cell epitopes (9mer peptides) in a given set of target HIV-1 sequences (87). Two recent studies in non-human primates demonstrated that vaccination with mosaic HIV-1 antigens resulted in broader and higher magnitude T cell responses than vaccination with natural antigens. Building upon the strategy of mosaic vaccines, polyvalent mosaic vaccines aim to incorporate cocktails of multiple complementary mosaic antigens into a single vaccine (87, 264). Complex computational algorithms are employed in order to significantly improve the population coverage of potential epitopes for HIV-1 proteins. While these approaches may provide for an extraordinary degree of
coverage of the potential HIV-1 strains that a vaccinee could be exposed to, they do not
preferentially focus the immune response against conserved epitopes, and hence are
unlikely to prevent immune escape.

In summary, were immunologists to eventually develop the means to focus T cell
responses against the determinants of their choosing, strategies could likely be devised
whereby HIV-1 could either be forced to a substantially reduced fitness, or cornered and
completely unable to escape from effective immune responses. In the meantime, the best
that we can hope for from our most advanced antigen-design platforms is that the T cell
response will recognize a founder population of transmitted virus. Perhaps if responses
could be generated of such a magnitude, durability and optimal localization that these
seeds of infection could be eliminated before infection became systemic, then such
responses would be of value. However, in the generally accepted mode of action of a
hypothetic T cell based vaccine, where systemic infection occurs but is durably
suppressed, what is required is the generation of T cell responses from which HIV-1
cannot escape. There is no viable strategy presently on the table which can offer to
achieve this goal.

In this thesis, I propose a radical new way of targeting T cell responses against
HIV-1-infected cells by eliciting responses which target stable surrogate markers which
are uniquely expressed upon cellular HIV-1 infection. These markers comprise the
human endogenous retroviruses and LINE-1 retrotransposable elements that reside in the
human genome. I was first led to this idea when I was performing routine sequencing of
HIV-1-gag from the blood plasma of an HIV-1-infected individual, and identified an
unusual sequence with a segment of LINE-1 inserted into HIV-1-gag. This sequence was missing many of the hallmarks of retrotransposition, such as target-site duplications, and I have not yet been able to either define its origin or to isolate similar sequences. It was, however, the catalyst which caused me to first pose the question – what is a LINE-1 element, and could it be somehow interacting with HIV-1?

1.4 - Type 1 Long-interspersed Nuclear Elements

1.4.1 - Biology and Replication of LINE-1 Elements.

One of the most striking discoveries resulting from the human genome sequencing project was the observation that 42% of our genome is comprised of retrotransposable element (RE) sequence (161). Long interspersed nuclear element 1 (LINE-1 or L1) elements represent the most prolific class of RE, and alone make up 17% of genomic sequences. An estimated 100 retrotransposition competent LINE-1 elements remain in the human genome, of which a small number (6 in the December 2001 freeze of the human genome working draft) are classified as highly active (40, 255). Intact LINE-1 elements are approximately 6kb in length and contain two ORFs. ORF1 encodes a 40 kDa protein with RNA chaperone activity, while ORF2 encodes a 150 kDa protein which possesses the endonuclease and reverse transcriptase (RT) activities required for retrotransposition (123, 152, 194). The LINE-1 lifecycle is depicted in Fig. 1.2. Retrotransposition is thought to occur by a mechanism termed target-primed reverse transcription (TPRT) where reverse transcription and integration are coupled as a single concerted step at the site of insertion (61, 138, 276).
Fig. 1.2. **LINE-1 retrotransposon replication cycle.** Transcription of full-length LINE-1 elements is initiated at a promoter in the 5’ UTR. The transcript is polyadenylated and exported from the nucleus. ORF1 is translated into a 40 kDa protein with RNA chaperone activity, while ORF2 is translated from an internal ribosome binding site into a 150 kDa protein possessing endonuclease and reverse transcriptase activity. The \(\text{cis}\)-binding activity of p40 for the RNA which encoded it catalyzes the formation of a ribonucleoprotein particle (RNP) comprised of p40, p150, and LINE-1 RNA. LINE-1 RNPs are imported into the nucleus by an unknown mechanism. Priming occurs against genomic DNA, frequently with the poly(A) tail of the RNA annealing to the rough TTT/A LINE-1 endonuclease cleavage site. Reverse transcription and integration occur as a single step termed target-primed reverse transcription (TPRT) resulting in generation of direct repeat, target site duplications that are the hallmark of LINE-1 insertions. Due to a poorly understood mechanism, the large majority of LINE-1 insertions are short 5’ truncated sequences.
1.4.2 – Expression and retrotransposition of LINE-1 in health and disease.

Initial evidence for the presence of retrotransposition competent LINE-1 elements in the human genome was provided by the discovery of de novo LINE-1 insertions in germ-line cells. Generally these insertions have been identified in cases where LINE-1 retrotransposition has resulted in the disruption of a gene, giving rise to an associated genetic disorder phenotype. Specific cases include insertion of a LINE-1 sequence into intron 5 of the X-linked gene CYBB resulting in aberrant splicing, and manifesting as chronic granulomatous disease, and an insertion of LINE-1 sequence into the 3’ end of exon 44 of the dystrophin gene resulting in a case of duchenne muscular dystrophy (201, 213, 217). LINE-1 retrotransposition in somatic cells has also been reported, and this likely contributes to some cases of carcinogenesis. This is highlighted by the identification of a LINE-1 insertion into the second intron of the myc oncogene in breast ductal adenocarcinoma tissue, which was absent in normal breast tissue from the same individual (209). Genomic instability induced by LINE-1 retrotransposition may also play a role in the progression of malignancies. A recent study found that LINE-1 promoter hypomethylation and associated transcription was significantly more frequent in blast-phase chronic myeloid leukemia (CML) than in chronic-phase CML, and that LINE-1 hypomethylation was prognostic of poorer progression-free survival (245). The activity of LINE-1 can therefore lead to genomic instability with substantial delitirious effects.

The roles, if any, of LINE-1 retrotransposons in normal human physiology are less clear. It has been argued that LINE-1 retrotransposition plays a role in the generation
of neuronal somatic mosaicism, the process by which neural stem cells differentiate into distinct lineages and cell types (63, 212). Initial support for this hypothesis was derived from the observation that engineered LINE-1 elements, containing a reporter gene expressed only upon retrotransposition, would retrotranspose upon transfection into neuronal precursors derived from rat hippocampus neural stem cells (212). It was further demonstrated that this LINE-1 retrotransposition could alter gene expression and affect cell fate. The same study demonstrated that, in mice which were transgenic for an engineered human LINE-1 element, retrotransposition occurred in germ cells and in the brain, but not in other somatic cells. Although these results are intriguing, their dependence upon artificial, engineered LINE-1 elements derived from another species necessitates that they be interpreted with caution. A second study from the same group sought to obtain more direct evidence for the retrotransposition of truly endogenous LINE-1 elements in human neural progenitor cells (63). Here it was demonstrated that neural progenitor cells isolated from human fetal brain support the retrotransposition of engineered human LINE-1 elements \textit{in vitro}. The group then analyzed levels of genomic LINE-1 DNA in various tissues and reported that genomic DNA from the hippocampus, and several other brain tissues, was enriched for copies of LINE-1. Although this evidence could be suggestive of \textit{in vivo} LINE-1 retrotransposition, the levels of LINE-1 DNA in brain tissues were at best 1.1x higher than in other tissues and thus are not entirely convincing. Conclusive studies on the roles of LINE-1 retrotransposition in normal human physiology are likely to be dependent upon the development of novel technologies which would allow investigators to precisely monitor the retrotransposition.
of truly endogenous elements. In the meantime, one piece of evidence argues against a vital role for LINE-1 retrotransposition *in vivo*. We have observed that nucleoside analogue reverse transcriptase inhibitors, used in the treatment of HIV-1, effectively suppress the retrotransposition of LINE-1 elements *in vitro*. The IC 50 values for stavudine (d4T), lamivudine (3TC) and zidovudine (AZT) in suppressing LINE-1 retrotransposition are achieved *in vivo* by standard dosing used in the treatment of HIV-1 (135). Were LINE-1 retrotransposition to play a critical role in normal physiology, one would expect that individuals treated with these drugs would exhibit substantial side-effects. For example, were LINE-1 retrotransposition critical to brain development, HIV-1-infected children treated with these drugs would be expected to be severely developmentally impaired – in particular for those treated with stavudine, which readily crosses the blood brain barrier. Thus at present, the cumulative evidence is ambiguous and a role for LINE-1 retrotransposition in normal physiology can neither be confirmed nor ruled-out.

In terms of our proposal to pursue LINE-1 protein products as antigenic targets for HIV-1 vaccines, it is critical to consider whether these proteins are expressed in healthy tissues. As with the potential for LINE-1 to contribute to normal physiology, this remains an area of controversy. Human ORF1p has been detected in a number of germ cell tumors, in breast carcinoma, medulloblastoma, and a variety of transformed cell lines (15, 35-37), there are only two reports of its expression in healthy tissues: one reporting expression in the epithelium of the mammary gland (15), and the second reporting expression in Leydig, Sertoli and vascular endothelial cells as well as placental syncytiotrophoblasts (80). Until recently, all efforts to detect LINE-1-ORF2p have failed.
Expression of LINE-1-ORF2p has now been reported in Leydig, Sertoli and vascular endothelial cells, in the same study reporting ORF1p expression in these same cell types (80). In our hands, early attempts to detect ORF2p expression by immunohistochemistry using crude polyclonal antibodies yielded similar staining patterns in the testis and vascular endothelium. Subsequent experiments using purified anti-sera resulted in more pronounced staining of positive control cells (transfected with ORF2), but a complete lack of staining of these tissues (manuscript in preparation). Thus, our overall interpretation of the data in the field leads us to conclude that ORF1p is likely to be expressed in some healthy tissues while ORF2p expression has yet to be demonstrated.

1.4.3 - Mechanisms of suppression of LINE-1 expression and retrotransposition.

Given the potential of LINE-1 elements to cause genomic instability, and their enormous prevalence in the genome, it is not surprising to note that host cells have developed multiple ways of tightly suppressing their activity. First amongst these is methylation of the CpG-rich LINE-1 5’UTR which allows for general repression in normal somatic tissues (306). For LINE-1 elements which are transcriptionally active, RNA-induced silencing is likely to play a role in preventing protein-level expression. The presence of forward and reverse oriented LINE-1 elements within transcribed genes is expected to produce a pool of double-stranded RNAs for siRNA production, as is the sense/anti-sense activity from the promoter of the LINE-1 5’UTR. Double-stranded small RNAs which hybridize to probes from the LINE-1 5’UTR have been detected, but not yet confirmed as bona fide siRNAs. Perhaps the strongest lines of evidence for a role for RNAi in the control of LINE-1 retrotransposition come from cell culture experiments,
where it has been shown that depletion of Dicer in cultured cells doubles LINE-1 retrotransposition (271). As a corollary, transfection of in vitro diced LINE-1 siRNA reduces retrotransposition of engineered LINE-1 elements in vitro (303).

Of particular relevance to this dissertation is the role of human APOBEC3 proteins in suppressing LINE-1 expression and retrotransposition. There are seven different APOBEC3 family members in primates, expanded from a single gene, at least five of which (3A, 3B, 3C, 3F and 3G) inhibit the retrotransposition of LINE-1 in cell culture. APOBEC3G was first identified through its ability to inhibit replication of HIV-1 mutants from which the vif gene had been deleted in a number of cell types (termed non-permissive) (268). The basis for restriction of HIV-1 by APOBEC3G is primarily through its deamination of minus-strand cDNA during viral reverse transcription – resulting in the heavy mutation of proviruses referred to as hypermutation (116, 165, 184, 311). There are other lines of evidence, however, which also implicate deaminase-independent effects on HIV-1 replication (29, 30, 111, 124, 128, 172, 270, 305). Interestingly, the suppression of LINE-1 retrotransposition by APOBEC3 proteins is not associated with hypermutation but rather is thought to involve sequestration of LINE-1 RNA in high-molecular-weight complexes away from the cellular translational machinery (51).

It has since been found that all seven APOBEC3 proteins are able to inhibit HIV-1 replication under certain conditions (reviewed in (51, 181)). In order to overcome these innate restriction factors, HIV-1 encodes the accessory protein Vif, which drives the ubiquitination and subsequent proteasomal degradation of several APOBEC3 family members including 3G, 3F, 3DE and perhaps 3H (56, 189, 200, 269, 273, 307). We
proposed that the degradation of APOBEC3 proteins by HIV-1-Vif would result in the aberrant expression and retrotransposition of the LINE-1 elements that those proteins normally help to suppress. Although we present this as one concrete example of a potential mechanism whereby HIV-1 infection could upset normal cellular control of LINE-1 elements, we feel that this more broadly illustrates the possibility for complex interactions between exogenous retroviruses and endogenous retrotransposable elements where diverse mechanisms may be at play.

1.5 – Human endogenous retroviruses (HERVs).

1.5.1 – Origin of human endogenous retroviruses.

The hallmark of a retrovirus is its ability to reverse transcribe an RNA genome into DNA and integrate this proviral DNA into the genome of an infected host cell. This copy of the retrovirus thus becomes an inseparable component of the host cell, and is passed on to all progeny when the cell undergoes division. In the vast majority of cases the infected cell is a somatic cell, such as a CD4+ T cell in the case of HIV-1. An infected host will therefore have only a subset of their cells containing integrated provirus, and this is generally a small minority of their total cells. Human endogenous retroviruses are established when a retrovirus infects a germline cell of a host and the acquired provirus is passed to offspring. In this manner, the provirus is integrated into every cell in the offspring and is then passed on in a Mendelian manner. Should the integrated provirus prove beneficial to the host, this endogenized retrovirus may come under positive selection and move towards fixation in the population. One notable example of a
beneficial role for an endogenous retrovirus is the human protein syncytin-1, which is the product of the env gene of a HERV-W provirus (33, 183). Here, the cell fusion properties of the retroviral Env protein are harnessed to mediate cell fusion and syncytium formation in the trophoblast cells that form the outer layer of the placenta. A number of other examples of endogenized retroviruses providing benefits to the host come from situations where ERVs protect cells from infection with closely related exogenous retroviruses (reviewed in (131). The sheep endogenous retrovirus enJSRV, for example, prevents replication of exogenous JSRV by expressing Gag proteins with dominant lethal mutations that prevent hybrid capsids from exiting the cell (228). In general, however, it is thought the large majority of HERV insertions in the human genome have very little selective consequence and have become fixed in the human population as a result of numerous genetic bottlenecks encountered by the human species over the past 100 million years (reviewed in (228)). The vast majority of HERV insertions in the human genome are thought to have been derived from a small number of germline colonizations which have since proliferated through either re-infection or retrotransposition (18). The net result is that the 7-8% of the contemporary human is comprised of HERV sequences. (72, 160)

1.5.2 – Families of HERVs and phylogeny.

The complement of HERVs in the human genome is incredibly diverse, and represents all three major branches of the retroviral tree: gamma-epsilon, spuma and delta-lenti-alpha-beta retroviruses. These HERV sequences paint a much more complete
picture of retroviral evolution than the relatively small number of extant exogenous retroviruses (Fig. 1.3)
Fig. 1.3. From (132). Representative unrooted Pol neighbour joining (NJ) dendrogram depicting diversity of endogenous retroviruses in humans and other species.
At present, there is no satisfactory or accepted standard for naming and classifying all HERVs. One of the more commonly used nomenclatures is tied to the tRNA complementary to the HERV primer binding site in the LTR. The designation of a HERV as HERV-K, for example, indicates that the primer binding site (PBS) binds to lysine-tRNA. This system can be misleading, however, as HERVs which are closely phylogenetically related may have differences in their PBS and, conversely, unrelated HERVs may bind to the same tRNA. Of particular relevance to this study, are a group of related HERV-K proviruses which have been given the designation human MMTV-like HML-1 through HML-10 due to their similarity with mouse mammary tumor virus (MMTV). In general, a more accurate and useful system of HERV nomenclature is urgently needed.

Although I am interested in the potential induction of the gamut of HERVs in the context of HIV-1 infection, the enormous diversity that this represents necessitated that we narrow our focus to a manageable subset. Four factors led me to select the HML-2 lineage of the HERV-K class II superfamily for further study. Firstly, although the first members of this lineage colonized the human genome 35 million years (Myr) ago, the presence of 73 human-specific HERV-K(HML-2) insertions demonstrates proliferation since the human and chimpanzee lineages diverged some 6 Myr ago. Furthermore, approximately 12% of 91 HERV-K(HML-2) loci are polymorphic amongst humans and are estimated to have inserted just 800,00 years ago, while the exceptionally preserved HERV-K(HML-2)-113 is less than 450,000 years old (275, 281). Owing to this relatively recent integration, HERV-K(HML-2) proviruses are comparatively intact and many
contain complete open reading frames for viral proteins (20, 26, 73, 115, 148, 179, 196, 211, 239, 280, 281, 301). Although no fully intact HERV-K(HML-2) pro-virus has yet been identified in the human genome, infectious HERV-K(HML-2) viruses can be reconstituted either from consensus sequences or by complementation between sequences from as few as three proviral loci (74, 167). The maintenance of long ORFs in this lineage provides a rich source of potential antigens that are amenable to study. Secondly, of all of the HERV lineages, HERV-K(HML-2) has been the most studied and is the best characterized. In addition to providing us with some valuable background knowledge, this has allowed for us to access HERV-K(HML-2)-specific reagents, such as antibodies, either commercially or through collaborations. Thirdly, several lines of evidence in the literature provided early support for the possibility of interactions between HIV-1 and HERV-K(HML-2), both through functional complementation of HIV-1 proteins by HERV-K functional homologues, and through the identification of host restriction factors with activity against both viruses (discussed below). Finally, we and others have previously reported the presence of HERV-K(HML-2) RNA in the blood plasma of HIV-1-infected subjects, suggesting that these elements may be induced in some form by HIV-1 infection (58, 96). Thus, while I do not wish to exclude the possibility that other HERV lineages may also be valuable targets, for the remainder of this dissertation I will focus upon the HERV-K(HML-2) lineage.
1.5.3 – Mechanisms of suppression of HERV-K(HML-2) protein expression and potential for disruption by HIV-1 infection.

Host cells have developed mechanisms to prevent lentiviral replication, as well as to restrict the expression of human endogenous retroviruses such as HERV-K(HML-2). These mechanisms include transcriptional silencing through DNA methylation, post-transcriptional silencing via RNA interference, post-entry interference by TRIM5α, and mutational inactivation of elements in the course of their retrotranposition cycle by cellular cytosine deaminases (81, 82). Likely due to successful implementation of these mechanisms in healthy tissues, protein-level expression of HERV-K(HML-2) is uniquely associated with disease states. In particular, the expression of HERV-K(HML-2) Gag and Env proteins has been associated with germ cell tumors and melanomas, and antibody responses to these HERV-K(HML-2) products are generally present at the time of tumor detection (43, 125, 185, 257, 258). We, and others have recently demonstrated that HIV-1 infection results in the upregulation of HERV-K(HML-2) RNA expression, the induction of HERV-K(HML-2) protein expression within infected cells, and the generation of an HERV-K(HML-2)-specific CD8+ T cell response (57-59, 96). The mechanism of HIV-1-induced HERV-K(HML-2) expression is presently unknown. One possibility is that the unspliced HERV-K(HML-2) RNA (such as that coding for Env and Gag), which is ubiquitously expressed in healthy cells, is strictly confined to the nucleus, thus preventing protein expression. The HIV-1 protein Rev has previously been demonstrated to facilitate the nuclear export of unspliced HERV-K(HML-2) RNA, and thus potentially grants HERV-K(HML-2) RNA access to the cytoplasm and hence to the cellular translational
machinery from which it is usually excluded (301). A second possibility is that the impairment of intracellular retroviral restriction factors by HIV-1 disrupts cellular controls normally responsible for suppressing HERV-K(HML-2) expression. The APOBEC3 family of cytosine deaminases, in particular 3F and 3G are known to suppress a broad spectrum of mammalian endogenous retroviruses, including HERV-K(HML-2) (82, 168). Of these, APOBEC3G and 3F proteins have also been demonstrated to be potent inhibitors of HIV-1 strains in which the accessory protein Vif has been deleted (165, 184, 268). HIV-1 Vif acts as a counter-measure against this innate antiretroviral defence mechanism by both preventing the translation of APOBEC3F and 3G, and inducing their proteasomal degradation (75, 189, 269, 273, 294). Given the demonstrated ability of APOBEC3F and 3G to suppress HERV-K(HML-2), it stands to reason that the disruption of the APOBEC3F and 3G proteins by HIV-1-Vif may allow for aberrant HERV-K(HML-2) expression.

1.6 – T cell exhaustion as a consideration in the development of therapeutic vaccination strategies:

We anticipate that initial human testing of a HERV-K(HML-2) or LINE-1 targeted vaccine would be performed in the setting of chronic HIV-1-infection, with the aim of eliciting enduring T cell mediated control of viral replication in the absence of antiretroviral therapy. In this setting, it will be important to take into consideration the impaired state of the immune system of the chronically infected individual. In HIV-1-infected individuals, as in other chronic viral infections, virus-specific T cells exhibit a
progressive loss of functionality termed T cell exhaustion (12, 14, 38, 67, 120, 232, 234, 265, 267, 278, 279, 310). As this phenomenon is driven, at least in part, by chronic exposure to antigens, we feel it is likely that HERV-K(HML-2) and LINE-1 specific CD8⁺ T cells will share this dysfunctional state. Recently, the molecule PD-1 has been demonstrated to play a causal role in the establishment of immune exhaustion in a number of chronic viral infections, including HIV-1 (19, 67, 90, 232, 233, 278). Blocking PD-1 signalling using antibodies has been shown to reverse this exhaustion both in vitro and in vivo. In Chapter 7 we present our work supporting a role for the molecule Tim-3 in the establishment and maintenance of an exhausted state. We envision the potential for combining therapeutic immunization targeting HERV-K and/or LINE-1 with an immunomodulatory agent aimed at reversing T cell exhaustion in order to expand functional HERV-K and/or LINE-1 specific CD8⁺ T cells in the context of chronic viral infection.
Chapter 2: Methods

2.1 – Subjects:
Subjects were selected from participants in the Canadian Immunodeficiency Research Collaborative (CIRC) Cohort, Toronto, Canada, and the OPTIONS Cohort, University of California San Francisco (UCSF). The CIRC cohort represented acutely/early HIV-1 infected subjects, HIV-1-infected chronic progressors, HIV-1-infected viremic controllers and HIV-1-infected elite controllers. Acute/early subjects were defined as individuals infected with HIV-1 within the last 4 months. Chronic progressors were defined as individuals infected with HIV-1 for > 1 year with CD4$^+$ T cell count decline >50 cells/mm$^3$/year. Viremic controllers were defined as individuals infected with HIV-1 > 1 year, no evidence of CD4$^+$ T cell count decline, and viral load <5,000 copies/ml bDNA. Elite controllers were defined as individuals infected with HIV-1 > 1 year with viral loads below the 50 copies/ml limit of detection (non-consecutive blips to < 500 copies/ml permissible) and no sign of CD4$^+$ T cell count decline. HIV-1 uninfected subjects were collected from the same demographic area, with a similar age and sex profile, and samples processed in an identical manner. OPTIONS Cohort: Baseline samples from all recruited subjects are evaluated to establish their HIV-1 infection status. Screened subjects must meet one of three criteria to be defined as having acute/early HIV-1 infection: (1) HIV-1 RNA >5,000 copies/ml with a negative or indeterminate HIV-1 antibody test, or; (2) a documented negative HIV-1 antibody test within 6 months with current seroconversion, or (3) a history compatible with acute/early HIV-1 infection with
laboratory confirmation based on a non-reactive less sensitive antibody test. All subjects discuss the advantages and disadvantages of early antiretroviral therapy with study staff and arrangements are made for therapy for those who elect to initiate treatment; slightly over half of participants decline therapy. All studies were approved by the University of Toronto Institutional Review Board and by the UCSF Committee on Human Research and subjects gave written informed consent.

2.2 – Sequences of primers, probes and plasmids:

**Table 2.1. Sequences of primers and probes.**

<table>
<thead>
<tr>
<th>Primer/Plasmid</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Probe (5’-3’)</th>
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</thead>
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<tr>
<td>HERV-K-HML-2-gag</td>
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<td>GAATTGGGAATGCGCCAGTT</td>
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<tr>
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<td>GGTGGTCTGAGGGTAGTAC</td>
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**Table 2.2. Sequences of plasmid standards for qPCR.**

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<td>GGGACTGTTAATGTCACACATT</td>
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GAGAAGTTTTAGTTTGGGAAGAATGTGTGCCAATAGTGCGGTGATA

HERV-K(HML-2)-pol
TAGAAAGTTGTTTACTCAGGGAATGCTTAAATAGTCGCCAAACTATTGTCAG
ACTTTTTTGACTCAAGGCTCTTCACCAGTTAGAGAAAGGAGTTTTCAGAC
TGTTATATCATCATTIGTGTTGAGATATTTTGTGTTG

HERV-K(HML-2)-env
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ACGTCGAAGAAGACAGATGGAAGTGGGCATCCACCAAGAAGGCGAGGCC
GCGCAGTTGGGACAACAATCAAGAAAGCTGAGCGCTGTAGCTCAAARA
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CGACGGTTAGTTGATGCAACTCAATGTTGGAAGTCTCCCTATGCGGTA
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TCGTTATAGTCTCTATGAGGAGGCTAGGACACCCAGAGGATTTTAACTG
TCGAGTCCAAAAATGTGTTGGAAGAATGTGTGCCAATAGTGCGGTGATA
2.3 – Quantitative PCR:

For both RNA and DNA quantitation amplicon identities were confirmed by sequencing.

2.3.1 – Quantitation of DNA (Chapter 3):

DNA was isolated using the Gentra Puregene genomic DNA isolation kit (Qiagen). Real-time PCRs were performed using the ABI Prism 7900HT (Applied Biosystems). At least
six replicates were performed for each sample. qPCR was performed using iTaq SYBR Green Supermix (BioRad) or Taqman Universal Mastermix (Applied Biosystems). Standard curves were generated using 10-fold serial dilutions of either gDNA (70ng – 0.7pg) or plasmid standards (109 – 103 copies). Human gene quantiations were performed using 7ng of sample gDNA, while LINE-1/Alu were quantified using either 7ng or 14pg of gDNA. 100ng of DNA were used in each of the eGFP and TBP absolute quantitation reactions. Reaction conditions for all experiments were: 95°C-3min, followed by 36 cycles of: 95°C-15s, 60°C-45s. For SYBR qPCR experiments, final dissociation stages were run to generate melting curves. Real-time PCR was monitored and analyzed with Sequence Detection System version 2.0 (Applied Biosystems).

2.3.1 - Quantitation of RNA (Chapter 4)

Primers and 5’-6FAM 3’-MGBNFQ probes (Applied Biosystems) for Taqman qPCR were designed with Primer Express (Applied Biosystems) using a HERV-K(HML-2) consensus sequence. To generate plasmid standards, gene segments containing the corresponding template sequence were synthesized and cloned into pUC57 (Genscript). These plasmids were linearized and diluted to single use stocks of 10⁹ copies/5ml. RNA was isolated from cells using either Allprep (Qiagen) or RNeasy Plus (Qiagen) kits following manufacturer’s instructions. PCR reactions were performed using Universal Taqman Mastermix (Applied Biosystems) with the following cycling conditions: 48°C – 15min, 95°C – 10min, 40 cycles of (95°C – 15sec, 58°C – 1min).
2.4 – Preparation of plasmid standards:
Gene segments containing the corresponding template sequence were synthesized and cloned into pUC57 (Genscript). These plasmids were linearized, diluted to $10^9$ copies/5ml, and cryopreserved as single-use aliquots.

2.5 – Generation of HIV-1 and HIV-2 viruses:
Primary isolate virus stocks were obtained from the NIH AIDS reference and reagent program (Table 4) and amplified on activated primary CD4+ T cells from an HIV-1-uninfected donor. The plasmids p197-1 and pNL4-3 were obtained from the NIH AIDS reagent program. Plasmid pNL4-3 encodes full-length infectious HIV-1-NL4-3 virus, while p197-1 encodes the 5’ portion of NL4-3 a deletion in vif (101). Vif-deleted virus was generated by digesting pNL4-3 with AgeI and EcoRI, and replacing the excised fragment with that derived from digesting p197-1 with the same enzymes. YU-2 and YU-2ΔVif were a gift from Michael Malim and have been previously described (268). Plasmids were transfected into 293T cells using Fugene 6 (Roche). Virus-containing supernatants were collected 4 days post-transfection and p24 concentrations were determined by ELISA (Zeptometrix). All viruses were purified by centrifugation through a 20% sucrose cushion.

Table 2.3. HIV-1 primary isolates

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### Table 2.4. Antibodies used in flow cytometry

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<td>IFN-γ-APC</td>
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<td>PD-1-APC</td>
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</table>
2.9 – Purification of high molecular weight genomic DNA:
5µg of total DNA was separated on a 0.8% agarose gel. High molecular weight DNA bands were excised and purified using the Puregene gel extraction kit (Sigma).

2.10 – Retrotransposition assay:
The in vitro LINE-1 retrotransposition assay is a modified version of a method which has been described elsewhere (117, 208, 224). Jurkat cells were maintained in RPMI-15% FBS. These cells were transfected with a plasmid containing a puromycin resistance gene and either the retrotransposition-competent human LINE-1 element LRE3 (99gfpLRE3) or an LINE-1 element that had been rendered retrotransposition incompetent by the introduction of point mutations in ORF1 (99gfpJM111) using 8µl of DMRIE-C Reagent (Invitrogen). Selection with 1µg/ml of puromycin was initiated at 48 hours post-transfection and continued for 28 days. Amounts of virus corresponding to 100ng p24 of NL4-3, or 200ng p24 of NL4-3-ΔVif were purified through a 20% sucrose cushion by centrifugation at 16,813xg for 1 hour prior to use in infections. Infections of 5x10⁵ Jurkat cells were performed for each virus, without a subsequent wash step, and aliquots were taken at indicated time-points. eGFP expression and intracellular HIV-1-Gag expression was analyzed by flow cytometry as described above.
2.11 – Targeted genomic difference analysis (TGDA) to identify novel LINE-1 insertions:

Template DNA was isolated from CD4+ T cells 168 hours post-infection with HIV-1-NL4-3 as well as from mock-infected controls. Preparation of tracer and driver DNA: Digestion of genomic DNAs, adapter ligation, and PCR amplification of LINE-1-flanking regions were performed as described (45). We amplified 1ng aliquots of DNA according to step-out PCR procedure with set A (0.01 µM A1A2, 0.2 µM A2, 0.2 µM L2) or set B (0.01 µM A2L2, 0.2 µM A2, 0.2 µM A1) primers for 15 cycles of the following: 95°C for 15 sec, 57°C for 10 sec, 72°C for 90 sec. We digested 150 ng each of the resulting tracer A and B samples, and 3000 ng of the mock amplicon (driver) with Exo III nuclease separately at 16°C using the following conditions: tracer A, 20 units of Exo III, 11 minutes (40 terminal nucleotides to be removed); tracer B, 20 units, 14 minutes (60 nucleotides to be removed); driver, 400 units, 11 minutes (40 residues to be removed). We mixed 15 ng each of the digested tracer A and B samples with 1500 ng of digested driver. DNA samples were purified by phenol/chloroform extraction, precipitated with ethanol, and dissolved in 3 µl of hybridization buffer (0.5 M NaCl, 50 mM Hepes pH 8.3, 0.2 mM EDTA). Suppression adapter sequences (5'-3') - Set 1: A1A2 (adapter oligonucleotide, 44 nt long) CTAATACGACTCATATAGGGCTCGAGCGGCCGCCCGGGGCAGGT, A3 (oligonucleotide complementary to the adapter 3’-terminal part, 10 nt long), GGCCCGTCCA, A1 (“outer” PCR primer, 22 nt long), CTAATACGACTCATATAGGGGC, A2 (“inner” primer for nested PCR, 22 nt long), TCGAGCGGCGGCCCCGAGGCAGGT. Set 2: B1B2 (adapter oligonucleotide, 43 nt long)
TGTAGCGTGAAGACGACAGACAGAAAGGGCGTGGTGCGGAGGGCGGT, B3
(oligonucleotide complementary to the adapter 3’-terminal part, 11 nt long),
GCCTCCCCGCCA, B1 (“outer” PCR primer, 21 nt long),
TGTAGCGTGAAGACGACAGACAGAA.

2.12 – Confirmation of novel LINE-1 insertions:
Sequences obtained from TGDA were analyzed by BLAST (www.ncbi.nlm.nih.gov/BLAST/) and BLAT (www.genome.ucsc.edu). Discontinuous alignments, where part of the input sequence aligned to a genomic locus while the rest aligned to different genomic locus/loci, were selected for further characterization (sequences SH2_D012 and SH4_P04). Primers were designed to sequences flanking the insertional junctions (i.e: one primer specific to LINE-1 3’ sequence and second primer specific for genomic insertion site). These primers were SH2_D012: D012abs-for 5’ctaattattctctcttaaatttcctttt3’ and D012abs-rev 5’tgtgcacatgtacactaaacctt3’, SH4_P04: P04abs-for 5’tgtgcacatgtacactaaacctt3’ and P04abs-rev 5’ggagggcggtctgtttctat3’. Using the genomic loci identified by UCSC BLAT, we also designed primers to target the expected pre-LINE-1-insertion genomic sequences of each insertion site. Since these primers spanned the putative insertion site, a LINE-1 insertion would disrupt the template resulting in either a larger than expected amplicon, or a lack of amplification. These primers were – SH2_D012: D012pres-for 5’tcagttttcaagtctgccttattgg3’ and D012pres-rev 5’gaataattgtttacttgcctt3’, SH4_P04: P04pres-for 5’tcagacagagacagcctaca3’ and P04pres-rev 5’ttggaagttctctcttt3’. Primers were tested for amplification of genomic DNA derived from uninfected cells of the same individual analyzed by TGDA, using
platinum Taq DNA polymerase (Invitrogen) following manufacturer’s instructions and using supplied buffers. 10ng DNA was used in each 50µl reaction, using 200nM each primer and the following cycle conditions: 95°C–5min, 40 cycles of: 95°C–30sec, 55°C–30sec, 72°C–90sec. SYBR qPCR was performed as described above.

2.15 – Immunohistochemistry:
4µm sections of paraffin embedded cell pellet or tissue were mounted on superfrost plus slides. Immunohistochemistry was performed using a Ventana XT autostainer (Roche Diagnostics). The protocol used included a heat pre-treatment (mCC1) before incubation with the primary antibody anti-HERV-K-Env (Austral Biologicals cat 1811) concentration 2.5µg/ml. A biotin donkey anti-mouse secondary antibody (Jackson Labs) was incubated for 30 minutes before incubation with streptavidin HRP and DAB substrate. A haematoxylin counter-stain was used to contrast the brown positive stain of the immunohistochemistry.

2.16 – Western blot:
Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) at a concentration of 50x10⁶ cells/ml. Proteins were separated by reducing SDS-PAGE using the NuPage system (Invitrogen) and transferred to PVDF membranes by standard methods. Membranes were blocked with 5% milk in PBS + 0.2% TWEEN-20 and probed with 1/5,000 dilutions of anti-HERV-K-Gag (HERM-1841, Austral Biologicals) or anti-HERV-K-Env (HERM-1811, Austral Biologicals). Membranes were then washed extensively and probed with a secondary goat anti-mouse HRP antibody (Jackson
Immunoresearch). After additional washes, membranes were treated with ECL substrate (GE Healthcare) and films were exposed.

2.17 – Design of overlapping peptide libraries and assembly into matrix pools:
The following HERV-K(HML-2) consensus sequences were used to generate a library of 15mer peptides overlapping by 11 amino acids:

>HERV-K(HML-2)-Env

MNPEMQRKAPPRRRRHRNRAPLTHKMNKMVTSEEQMKLPSSTKKAEPPTWAQLKKLTQLATKYLENTKVTQTPESMLAALMIVSMVSLPMPAGAAANYTYWAYVPFPPLIRA
VTWMDNPIEYVVNDSWGVPIDDRCPAKPKEEGMMINISIGRYPPICLGRAPGCLMPAVQNWLVEVPVTSPICRFTHVMSGMLRPRVNYLQDFSYQQRSLKFRPKGKPCPKEIPKES
KNTEVLVWEECVANSAVILQNNETFIIIDWAPRQFYHNCYGQTQSCPASQVSPAVSD
LTESLDKHKKKLQSFYPWEGKGIUPRIPKVSPVSGPEHPPELWRHTAVSHHRIWSG
NQTLETDRKPYTIDLNSSLTVPLQSCVKKPIYMMLVGNIVIKPDSQITICTCENCRLTICIDS
TFNWQHRILLVRAEGRVWIPVSMRPEASPSVHILTEVLKGVLSRKRFITFTLIAVIMG
LIAVTATAAVAGVALHSSVQVSVFVNDWQKNSTRLWSQSSISDKLANQINDLRQTVI
WMGDRLMSEHRFQLQCDWNTSDFCITPQIYNESEHHWDVMVRHLQGREDNLTDISK
LKQEIQFASKAHNLVPGTEAIAGVADGLANLPVTWVTIGSTTTINLILILVCLFCLLLV
CRCTQQLRRDSDHRERAMMTAMVLKSKRGGNVKSKRDQIVTVSV
MGQTKSKI KSKY ASYL SFI KALLRKGVVKSVTNKLKLQFQIIEQFCPWPEQGTMLDLKDWKRI GELKQAGRKNIIPLTVNWDAIKAALPEFQTEEDSISYSDAPGSCII DCNENTRKRKSKKTESLHCEYVAEPVMAQSTQNVYNQLEQEVETLKLK EGV GELVGPSERGPTGSLPAGQPVPVTLQPQKVKENKTOPPVAYWQPLAQYR PPSQEQYGPAPQAPGAPRPSYQPPTRNLNPTAPSRQGSELHEIDIKRSKEDT EAQWQFVPTLQMPPPGGAQEPEQEGPTEVEARYKSFSIKMLKDMKEGVKQYPNSP Y MRTLDDLIAHGRILIPYDWEILAKSSLSPSQFLQFTWIDQVQEQVRRNAA NP PVNIDADQLLLGIGQWSTIQAMQNEAIEQVRAICLRAEKIQDPGSTPSFN TVRQGSKFLPYPDFVARLQDVA KSIADARKVIVELMAYENANPECQSAIKPL KGKVPAGSDVISEYVKACDGGTMLMAQAITGGLGQVRTYGGKCY NCGQIGHLKKCNCPVLNKQNITIQATTTGREPPLCPCKKGKHWASQCRSKFDK NGQPLSLGNEQRGQPAPQPTQTGAFIPIQFVPQPQGQPPPLSVFQGISQLPQYNN CPPQAANVQQ>

>LINE-1-ORF2p

MTGSNSHTILTLNINGLSIAKRHRLLASWIKSQDPSVCCIQETHLTCRDTHRLKIK GWRKIYQGANGKQKAGVAILVSDKTDFTKPDKTIKRDKEGHYIMVKGSIQQEELTIL NIYAPNTGAFFRPIQKQLSDLQRDSLRTLIMGDFNPLSTLDRSTRQKVNKDTEQ LEINSAHQLADIDYRTLHPKSTETYFFSAPHTHTSYKIDHIVGSKALLSSKCRTEIT NYLSDHSAIKLERLIKLQTSRPTTWTNLNLNDYWHNMKAIEKMMFETNENKDTYYQNLDFAKAVCRGKFIALNAYKRKQRKSKIDTLTSQLEKELKOEQTHS KASRRSEQEITKIRAEKLEIETKQLTQKINESRWSFFERIKIDRPLARLIIKKKREQNI DTIKNDKGITDITEDPQEITQITIREYKHTLYANKLENLEEMDTFLDTTYLPLRNQEEV ESLNRPITGSEIVAIINSLPTKSPGGDFTAEFYQRYKELVPFLLKLFQSIIEKEGIL PNSFYEASILIPKPGRDTTKEKFNFRPIMLMIDAKILNKILANRIQHHKLIHHDQ VGFPIMQGouflRNSINVIQHINAKDKNHMISDAEIKAFDKIQPPFMLKTLNK LGIDGTYFKIIAYDAPTNIQLNQGKLEAFPKTKTGRQGCPQLPFNLQEEVLAIR OQEEKGIQLGKKEVVKLSLFADDMIVYLENPIVAQNNLKLISNFKSVGYSYKIN VQQSAFQLYTNRQTESQIMGELPFITAIASKRKYLGQLIQTLRDVKDLKENYKPLKL EIKEETNWKKPNCSVWGRINIVKMAILPKVIYRFNAIPKLPMTTTELETTLKF1 WLNQKRARIASKS4QKNNKAGGISITPDFKLYYKATVTKTAWKYWQNRDIDQWNRT EPESEIMPHYNYLDFDKPEKNKQWGKDFSFLNWCWENLALCRKLKDLPFFTP YTKINSRWIKDLNKPKTIKTLLEENLITIQDIDVGKDFMSKTPAMATDKIDK WDLIKLKSFTAKETTIRVNRQPTTWKEIFATYSSDKGLISRIYELKQYKKTNN PIKKWWAKDMLHRFSEKIYAYAAKHMKKCCSLSLAIREMQIKTTMRYHTPVRMA IAIKKGSSNRCWRCGEGI4TLLICWWDCKVLQPLWKSVWRFLRLDELLEIPFDP AIPPLGIPNEYKSCYKDTCRMFIAALFTIAKTXIWNPQKCPMTIDWIKKMWHIYT MEYYYAIKNDDEFISFVGTWMKLETIILSKLSQQEQKTKHRIFSLIGN
Peptides were synthesized at 20mg scales by JPT Peptide Technologies (Berlin) and pooled as indicated in the peptide matrix below – where HKEnv pools 1 – 13 contain each of the peptides in the corresponding column while HKEnv pools 14 – 27 contain each of the peptides in the corresponding rows.
Matrix pools were dissolved to a concentration of 2mg/ml/peptide in 100% DMSO (Hybri-Max, Sigma), diluted to a working stock of 200µg/ml/peptide in 10% DMSO and used in ELISpot assays at a final concentration of 10µg/ml/peptide in 0.5% DMSO.
2.18 - IFN-γ ELISPOT screening.

Cryopreserved PBMC were resuspended in AIM-V medium (Invitrogen) supplemented with 60 U/ml benzonase (to prevent clumping) at a concentration of 2x10⁶ cells/ml and placed in the incubator overnight. Multiscreen IP 96-well plates (Millipore) were coated with 50µl/well of sterile PBS + 15µg/ml primary anti-IFN-γ antibody (clone 1-D1K, Mabtech) for 1 hour. Plates were washed with 3x 200µl sterile PBS then blocked with 40µl/well of AIM-V medium for 30 minutes. PBMC were counted, resuspended at 1.34x10⁶ cells/ml in AIM-V medium and added to plate at 150µl/well. Peptide matrix pools (200µg/ml/peptide stock in 5% DMSO) were added at 10µl/well to give a final concentration of 10µg/ml/peptide in 0.25% DMSO. SEB was added as a positive control to a final concentration of 10µg/ml. Plates were incubated at 37°C, 5% CO₂ for 16-18 hours. Cells were then flicked out of plates and plates were washed 4x with 200µl PBS + 0.1% TWEEN-20 (PBST). Secondary antibody – clone 7-B6-1 (Mabtech) was diluted to 1µg/ml and added at 50µl/well. Plates were incubated with secondary antibody for 1 hour. Antibody was then flicked out of plates and plates were washed with 4 x 200µl PBST. Steptavidin-ALP (Mabtech) was prepared at 1µg/ml in PBS and added to plates at 50µl/well for 1 hour. Tertiary antibody was removed from the plate and washed with 6 x 200µl PBST. Colour development solution: 9.4ml ddH20, 500µl 25x AP Color Development Buffer (Biorad), 100µl Color Reagent A (Biorad), 100µl Color Reagent B (Biorad) was added to the plate at 100 µl/well. Development was allowed to proceed for 10 minutes. Plates were then washed with water, dried overnight and counted. A positive
response was considered as one which met both of the following two criteria: 1) >50 sfu/million PBMC after background subtraction 2) >3x above background.

2.19 – Confirmation of responses.
A HERV-K(HML-2) or LINE-1-specific T cell response identified by peptide ELISPOT was not considered valid until it had been: 1) confirmed with an individual peptide 2) repeated with a newly synthesized batch of peptide from a separate supplier. The requirement for a separate supplier emerged after a particularly insidious case of contamination where we thought we had confirmed a response with a new batch of peptide, only to find that the manufacturer had shipped us left-overs from our original order for our second order.

2.20 – T cell cloning.
PBMC were stimulated for 6-10 hours with 10-100 µg/ml of HERV-K(HML-2)-derived peptides. For CMV-pp65 and HIV-Gag specific T cell cloning, cells were stimulated with peptide pools comprising 1 µg/ml/peptide. PBMC were enriched for cells that had produced IFN-γ in response to these peptides using the IFN-γ secretion assay – detection and enrichment kit (Miltenyi Biotec), following the manufacturer’s instructions. Enriched cells were plated at serial dilutions on a standard irradiated feeder cell medium: 1x10^6 allogeneic PBMC/ml, 1x10^5 cells/ml each two allogeneic BLCLs (all irradiated at 5000 rads) in RPMI 10% FBS (Gibco), 50 U/ml IL-2 (Hoffmann La-Roche), 1µM nevirapine, 1µM AZT, 1µM 3TC (NIH AIDS Reagent Program). Cells were cultured at 37°C, 5% CO₂ for 14 days, and then fed with 50µl/well RPMI 10% FBS, 50 U/ml IL-2, 1µM
nevirapine, 1µM AZT, 1µM 3TC (to prevent any possible outgrowth of HIV-1). Plates were monitored for an additional 14 days, and wells exhibiting growth were fed as above when media yellowed. On day 28 post-plating a plate was selected which exhibited growth in < 1/10 of wells. Putative clones were screened for specificity by IFN-γ ELISPOT using autologous BLCLs pulsed with corresponding peptides (Austral Biologicals). Specific clones were expanded in 1ml of feeder medium (see above) in 24 well plates, and subsequently re-stimulated at 3 week intervals.

2.22 – Assaying T cell recognition of HIV-1-infected CD4+ T cells.
T cell clones, taken at least 2 weeks after restimulation, were co-cultured with autologous infected or uninfected (mock) CD4+ T cells in the presence of 50U/ml IL-2, a 1:40 dilution of anti-CD107a-PE antibody (Biolegend), 1µg/ml Brefeldin A (BD) for 6 to 12 hours. In MHC-I blocking experiments anti-HLA-A,B,C (clone DX17, BD) and isotype controls were used at 10µg/ml. Cells were then surface stained with anti-CD8-FITC (Biolegend), permeabilized, and stained intracellularly with anti-IFN-γ-APC (BD) using standard methods. Cells were fixed in 5% formalin and analyzed on a FACSCalibur instrument. Data analysis was performed using Flowjo (Treestar).

2.23 – HIV-1-infected cell elimination assay.
T cell clones, taken at least 2 weeks after restimulation, were co-cultured with infected or uninfected (mock) autologous CD4+ T target cells at a ratio of 1 clone : 10 target cells for 24 hours in the presence of 50U/ml IL-2. Cells were stained with anti-CD8-Fitc and anti-CD4-APC (Biolegend), permeabilized, then stained intracellularly with anti-HIV-Gag-PE
(Kc57-RD1, Beckman Coulter). Cells were fixed in 5% formalin and analyzed on a FACSCalibur instrument. Data analysis was performed using Flowjo (Treestar).

### 2.24 – HIV-1 viral suppression assays

CD4⁺ T target cells were infected with 0.01 MOI of an HIV-1 primary isolate and plated at 50,000 cells/well in RPMI + 10% FBS + 50U/ml IL-2 in a 96 well plate. Clone cells were added at indicated effector:target ratios and co-culture was allowed to proceed for 9-16 days. Supernatants were harvested and assayed for p24 by ELISA following manufacturer’s instructions (p24 Antigen Detection Kit, NCI Frederik).

### 2.25 - siRNA HERV-K-env knockdown

siRNAs were obtained from Dharmaco: HERV-K(HML-2)-env siRNA duplex 1: 5’-GAAGAAGGGAUGAUGAUAAUU-3’, HERV-K(HML-2)-env siRNA duplex 2: 5’-AAGAAGGGAUGAUGAUAAAU-3’, On-TARGETplus Non-targeting siRNA #1 (Cat# D001810-01-05): 5’-GGUUUACAUGUCGACUAA-3’. Cells were electroporated with 165nmol of RNA using the human T cell nucleofector kit (Amaxa). Cells were then magnetofected with HIV-1 and, 16 hours later, were used as target cells in recognition assays following our standard method.

### 2.26 - Transfection of primary CD4⁺ T cells with HIV-1-vif

Codon-optimized vif from the primary HIV-1 isolate ‘ELI’ was synthesized by Genscript (genbank accession GU945071) and cloned into pGEM4Z/GFP/A64 (31). Linearized pGEM4Z/ELI-vif/A64 was used as template for in vitro transcription (mMessage mMMachine T7 kit, Ambion). mRNA was treated with DNase (TURBO DNA-free,
Ambion), and isolated using MegaClear (Ambion). Primary CD4\(^+\) T cells were transfected with 2\(\mu\)g of mRNA using the human T cell nucleofector kit (Amaxa). Transfection efficiency, assessed by GFP expression, was consistently found to be >80%. Expression of Vif was confirmed by western blot probing with 1:1,000 Vif antiserum (NIH AIDS reagent program, cat #2221).

2.27 - T cell receptor sequencing.

1x10\(^6\) cells of the HERV-K(HML-2)-Env-specific clone were lysed in RLT buffer (Qiagen) and homogenized with a blunt 20-gauge needle fitted to an RNase-free syringe. RNA was isolated using the RNeasy kit (Qiagen) following the manufacturer’s instructions. cDNA was synthesized using the SMARTer RACE cDNA amplification kit (Clonetech). 5’-RACE was performed using a gene-specific primer (GSP) on the 3’ end and the adapter binding sequence on the 5’ end added by the kit. For alpha chain amplification, a single 3’ GSP was used: Alpha - 5’CAGCAGTGTTTGGCAGCTCT3’. For beta chain amplification, a set of 2 GSP 3’ primers were used to target the 2 different possible constant regions: Beta-1: 5’AGA AGCGCTGGCAAAAGAAG, Beta-2: 5’CAGGAGAATCCTGGGTGAG. Amplification was performed by touchdown-PCR with the following cycling conditions: 1) 5 cycles of 94°C – 30 sec, 72°C – 3 min 2) 5 cycles of 94°C – 30 sec, 70°C – 30 sec, 72°C – 3 min 3) 30 cycles of 94°C – 30 sec, 68°C – 30 sec, 72°C – 3 min. This strategy was designed so that during the first 10 cycles of amplification GSP primers would bind preferentially to the template, while in the last 30 cycles the universal primers would bind to the adapter. Nested PCR was performed using
a nested GSP on the 5’ end, and a nested primer for the adapter sequence (provided by the Clonetech kit). As before, one primer was used for alpha: Alpha-internal – 5’TGTCAGGCAGTGACAAGCAG while two primers were used for beta: Beta-internal – 5’TGGGATGCAGAGGAGGTGAGA and Beta-2-internal – 5’AACCAGGCCCCAACCACACAAT. Second round PCR products were visualized on a gel. A product of the expected size (1kb) was cut out using the GeneJET Gel Extraction kit (Fermentas) following manufacturer’s instructions. The extracted DNA was TOPO cloned using the Invitrogen pCR 4-TOPO vector TA cloning kit in TOP10 cells. TOP10 cells were selected on amp+ plates and selected colonies were grown up and mini-prepped. Sequencing was performed using TOPO vector primers: M13-forward and M13-reverse using an ABI 3730 instrument. Independent colonies were sequences to confirm results. V/J cassettes were determined using the IMGT/V-QUEST bioinformatic tool at http://www.imgt.org/IMGT_vquest/share/textes/.

2.28 – Assaying T cell recognition of HIV-1-infected macrophages.

Monocytes were isolated as below (2.29.1) and differentiated into macrophages by culture in RPMI + 10% FBS supplemented with 10ng/ml of M-CSF (R&D systems). Half volume medium changes were performed on days 2, 4 and 6. Cells were harvested by treatment with cold PBS + 2mM EDTA and washed twice. These macrophages were then infected with the CCR5-tropic stain of HIV-1 ‘BaL’ and recognition assays were performed as with CD4+ target cells (see 2.22).
2.29 – Expansion of HERV-K(HML-2) and LINE-1 specific T cells using mRNA transfected dendritic cells (DCs).

2.29.1 – Isolation of monocytes and differentiation into dendritic cells.

100x10^6 cryopreserved PBMC were thawed from each subject to be tested. Monocytes were enriched from these PBMC by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec) following the manufacturer’s protocol. This typically yields a monocyte cell count of 10% the total number of starting PBMC. Monocytes should appear large (as compared to lymphocytes) and round. Enriched monocytes were stained with fluorochrome-conjugated antibodies to CD3 and CD14 to check purity. Monocytes are CD14^+; lymphocyte contaminants will show a population of CD3^+ T cells. A monocyte purity of >85% viable CD14^+ cells was required in order to proceed to DC differentiation. Monocytes were cultured at 1x10^6 cells/ml in DC media (CellGenix + mM L-glutamine + 800 U/ml GM-CSF (R&D systems) + 1,000 U/ml IL-4 (R&D systems) + 100uM Nevirapine (to prevent any outgrowth of HIV). Typically, cells are plated in 6 well plates with 2-3ml of medium per well. If less DCs are obtained they can be plated in 12 or 24 well plates. Three days following initiation of DC differentiation an additional 800 U/ml GM-CSF and 1,000 U/ml IL-4 were added without changing the medium. Differentiated DCs were harvested on day 5 of culture and stained with fluorochrome conjugated antibodies to CD86 and CD14. DCs will have downregulated CD14 and upregulated CD86 as compared to monocytes. If, for any donor, DC viability was <50% and/or viable cells were <80% CD14^lowCD86^hi then experiment was discontinued for this donor.
2.29.2 – Production of HERV-K(HML-2) and LINE-1 poly(A)-tailed mRNA.

Codon-optimized HERV-K(HML-2) gag, env, rec and LINE-1-ORF2 were provided by Pfizer Inc. These coding sequences were cloned into the vector pGEM4Z/GFP/A64 at the HindIII/EcoRI sites (excises GFP) (31). The sequences of these constructs were confirmed, plasmids were linearized with the restriction enzyme SpeI, precipitated, and then used as a template for in vitro transcription (mMessage mMachine T7 kit, Ambion). The resulting mRNA was treated with DNAs (TURBO DNA-free, Ambion), and isolated using Megaclear (Ambion).

2.29.3 – Electroporation of dendritic cells and maturation.

DCs were split into equal aliquots depending on number of antigens to be tested (as well as mock transfected control). The minimal number of DCs used for a given transfection was 2x10^5. Cells were pelleted and resuspended in 20µl of nucleofection solution from the Monocyte Shuttle Nucleofector kit (Amaxa). Cells were mixed with 2µg of mRNA, transferred to 96-well nucleofection plates and transfected on the Amaza shuttle system using program CB-150. Alternatively, nucleofections were performed using the Amaza 4D system using solution P3 and program C4. 100µl of DC medium was added to wells on nucleofector plate, and cells were gently transferred into a 24 well plate at a concentration of 1x10^6 cells/ml in DC maturation medium. DC maturation medium consists of DC medium (CellGenix) + 800 U/ml GM-CSF + 1,000 U/ml IL-4 +10ng/ml TNF-α + 10ng/ml IL-1β (all from R&D Systems) + 100 ng/ml Nevirapine (NIH AIDS reagent program) + 1µg/ml prostaglandin E2 (Sigma). Maturation was allowed to proceed overnight. The following day, DCs appeared large, uniformly round, and had
formed into blasts. Cells were checked for GFP expression by flow cytometry and >50% viability with >50% of viable cells expressing GFP was required to proceed with co-culture.

2.29.4 – CFSE labeling of PBMC

PBMC autologous to DCs were thawed (require ~2x10^6 viable PBMC for a typical expansion. PBMC were labeled with CFSE by washing in 10ml PBS, resuspending in 10ml PBS + 10μM CFSE and washing in 5ml RPMI + 10% human AB serum. Labeled PBMC were cultured at 1x10^6 cells/ml in RPMI + 10% human AB serum + Glutamine + pen-strep overnight.

2.29.5 – Co-culture of PBMC with nucleofected dendritic cells.

Mature DCs were washed with 3 x 14ml of 1% human AB serum in PBS removing as much supernatant as possible with each wash (to remove inflammatory cytokines). DCs and PBMC were combined in co-culture medium (RPMI + 45% Clicks medium + 2mM GlutaMAX TM-I + 5% human AB serum + pen-strep + 1μM Nevirapine) at final concentrations of 3x10^6 PBMC/ml + 1x10^6 DCs/ml.

2.29.6 - Assessing expansion of antigen-specific T cells by flow cytometry

Proliferation of T cells in response to mRNA-transfected DCs was assessed by monitoring CFSE diminution by flow cytometry. A response was considered to be positive if it met both of the following criteria: i) >2x level of background proliferation (in response to mock transfected DCs) and ii) >2% CFSE dim after background subtracted.
2.29.7 – Recognition assays using dendritic-cell expanded cell lines.

T cell lines, expanded with transfected DCs, were tested for recognition of HIV-1-infected cells using the same methods used to test T cell clones (see 2.22).

2.30 – Statistical Analyses.

The statistical tests used for a given analysis are indicated in the corresponding figure legends. Most statistical tests were performed using Prism Graphpad software. We used mixed effects longitudinal analyses to determine if CD8\(^+\) T cell activation levels independently associated with Tim3 percentage on CD8\(^+\) T cells during anti-retroviral therapy. We specified a random effect for time and the individual. The models were run in the SAS System 9.2 under Proc Mixed.

2.31 - Pentamer Analyses.

All pentamers were obtained from Proimmune Ltd. Cryopreserved PBMC samples from chronically HIV-1 infected HLA-A*0201\(^+\) individuals were thawed, and washed with 2 x 10 ml of 1% FBS PBS with 2mM EDTA. Staining was performed immediately after thawing with fluorophore conjugated antibodies against CD8 (BD), Tim-3 (R&D Systems), CD3 (BD), and the indicated pentamers (unlabeled), followed by a secondary staining step with APC labeled pentamer fluorotags. Cells were washed 2 x with 1% FBS PBS, and then fixed in 2% paraformaldehyde. Analysis was performed using a FACSCalibur instrument (BD Biosciences).
2.32 - Synthesis of recombinant Tim-3.

The expression vector, pPA-TEV, was previously derived from pIRESpuro3 (Clontech), and modified to incorporate the transin leader sequence and N-terminal Protein A tag. The Tim-3 insert was obtained from PCR using the following primers Tim-3-extF 5’ TTCGGCCGGCCCTCAGAAGTGAATACAGAGCGG 3’, Tim-3-extR 5’ TGAGCGGGCCGTCCATCATCTGATTGTTGCTCCAGAGTC 3’. For each primer the underlined bases represent the template annealing sequence. Additional 5’ sequences comprise restriction sites and stop codons. The region amplified by these primers constitutes only the IgV and mucin domains of Tim-3. The resultant Tim-3 amplicon was cloned into the Fse I/Not I cloning site of pPA-TEV. 10 µg of circular DNA plasmid was then transfected into HEK293T cells using the calcium phosphate method (Invitrogen). Expression of Tim-3 was confirmed by Western blot using a 1/5000 dilution of a polyclonal anti-Tim-3 antibody (R&D Systems) and a 1/5000 dilution of HRP-conjugated streptavidin (Pierce). Transfection was then repeated with linearized pPA-TEV-Tim-3 plasmid to generate stable cell lines. A parallel transfection was performed with empty linearized pPA-TEV. Three days after transfection, puromycin drug selection was initiated by replacing the media with fresh media supplemented with 1 to 5 µg/ml puromycin. The media was exchanged with fresh puromycin containing media every 2 days. Ten days later 6 colonies from the pPA-TEV-Tim-3 transfection, and 6 from the pPA-TEV transfection were isolated and expanded into 6 well tissue culture plates. Secreted proteins were detected by Western blot analysis using an anti-Tim-3 antibody for pPA-TEV-Tim-3, and an anti-protein-A antibody for pPA-TEV. A Tim-3 secreting
clone (pPA-TEV-Tim-3 transfected), and a control protein A secreting clone (pPA-TEV transfected) were selected and grown up in 2 L each of CHO-SFM-II media supplemented with 2% FBS, penicillin, streptomycin, HEPES, L-glutamine, and 1ug/L apoprotinin (Sigma) in 6, T175 tissue culture flasks. Cells were plated at 50% confluency, and protein secretion was allowed to continue for 5 days. Supernatants were concentrated from 2 L to 10 ml using centricon plus 70 centrifugal filter units (Millipore). Proteins were purified using IgG Sepharose 6 Fast Flow beads (GE Healthcare) as per the manufacturer’s instructions. 200 µl of 0.33 mg/ml His-tagged TEV protease were then added to the beads, and cleavage was allowed to proceed overnight at 4°C. Supernatants were removed from beads, the beads were washed 3x with 1 ml of TST, and supernatants were pooled with wash eluates. This combined eluate was passed through a 1 ml nickel column (B-PRE 6xHis fusion protein purification kit, Pierce) to remove TEV protease, and washed with 3 x 2 ml of wash buffer 2 from the same kit. The eluates were subsequently passed through detoxi-gel endotoxin removal columns (Pierce) following manufacturer’s instructions, and then concentrated to 0.5 ml using centricon plus-20 centrifugal filter units (Millipore). Volumes were then adjusted to 15 ml using sterile PBS, and reconcentrated to 0.5 ml. The purity and identity of products were confirmed by SDS-PAGE and Western blot analysis. Protein concentration was determined by a Bradford assay. As expected, only small amounts of residual protein were detectable in the protein-A control purification. This sample serves as a control for any effect of contaminant proteins, or reagents from the purification process on proliferation or cytokine production.
2.33 - Peptides and stimulation reagents.
Overlapping HIV-1 Clade B Gag and Nef pooled peptides (10mg/ml) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD). CEF (human Cytomegalovirus, Epstein Barr and Influenza Virus) pooled peptides (10mg/ml) (Anaspec), SEB (Sigma), and purified anti-CD3 and anti-CD28 monoclonal antibodies (BD) were used as additional reagents.

2.34 - Signaling Analyses.
Prior to analyses of cellular signaling, archived PBMCs that had been viably frozen were thawed in 15 mL RPMI cell culture medium (Mediatech) containing 5% FBS (HyClone; RPMI+), washed in PBS containing 2% FBS (PBS+), and then rested at 5x10⁶ cells/mL in RPMI+ at 37°C, 5% CO₂ over night. The following day, cells were washed with ice-cold PBS+, transferred to a 96-well V-bottom plate and stained for cell surface markers with fluorophore-conjugated monoclonal antibodies against CD3, CD8, CD27, CD45RA and Tim-3, on ice for 40min. An amine-reactive dye (Invitrogen) was used to stain dead cells. After washing, cells were transferred to PBS containing IL-2 (SIGMA; final 100ng/mL), or combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (P+I) (SIGMA; final 100 ng/mL and 1 μg/ml, respectively) at 37 °C to induce signaling. Signaling was arrested after 15, 30 and 45min by immediate fixation, adding 4% paraformalehyde (final concentration 2%). After 20 min fixation and subsequent washing, cells were permeabilized in 70% ice cold methanol for 20 min on ice. Cells were washed and stained with an antibody cocktail containing phospho- specific antibodies: p-Erk1/2(pT202/pY204), p-p38(pT180/pY182) and p-Stat5(pY694) (BD) for
60 min on ice. Before analysis, cells were washed and resuspended in PBS+ with 0.05% formaldehyde. The unstimulated control cells underwent the same manipulations. Cells were analyzed on a customized LSR II Flow Cytometer (BD). Analysis of data was performed using FlowJo (Tree Star). Fold changes in phosphorylation were calculated as the ratio of Median Fluorescence Intensity (MFI) of stimulated cells over unstimulated cells.

2.35 - Proliferation assay (for Tim-3 study).
To track cell division, PBMC from chronically HIV-1 infected individuals were labeled with 1μM of the fluorescent intracellular dye, 5-(and -6) carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes) in PBS and mixed periodically for 10 minutes at room temperature. Labeling was quenched by addition of an equal volume of complete media (15% FBS in RPMI) for 2 minutes. The labeled cells were then washed twice, counted and resuspended in cell culture media. CFSE labeled cells were stimulated for 5-6 days with either DMSO alone, SLYNTVATL peptide, pooled HIV-1 derived Gag and Nef peptides or Cytomegalovirus, Epstein-Barr Virus, and Flu Virus (CEF) pooled peptides in the presence or absence of either sTim-3 or an equal volume of expression control. At the end of the culture period, cells were washed and incubated with a combination of the following conjugated anti-human monoclonal antibodies: CD4, CD8 (BD Biosciences, San Jose, CA), and. Intracellular staining for (IFN-γ, IL-2 (BD, San Diego, CA) and CD3 (Beckman Coulter, Fullerton, CA) was performed after cells were fixed and permeablized. Cells were then washed in PBC with 2mM EDTA and 1% bovine serum albumin and then fixed in 1% paraformaldehyde before being run on an
LSRII flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed by using Flowjo Software version 6.4 (Treestar Inc, Ashland, OR).

2.36 – Biosafety.
Experiments involving the generation or manipulation of HIV-1 viral stocks or cells infected with exogenous HIV-1 were performed under biosafety level III conditions. All other tissue culture was performed under biosafety level II conditions. Molecular biology work involving fixed or lysed samples was performed under biosafety level I conditions.
Chapter 3: Induction of LINE-1 retrotransposition in HIV-1-infected cells

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Contributions: The experiments displayed were performed by Brad Jones, with the following exceptions. FACS in Fig. 3.2A was performed by Lishomwa Ndhlovu. TGDA in Figs. 3.4, 3.5 was performed by Anton Buzdin. The retrotransposition assay in Fig. 3.7B was performed by Haihan Song and Yang Xu, as was the flow cytometry in Fig. 3.7D. Haihan Song and Yang Xu also contributed to Fig. 3.7C, E and F (performed the transfections while Brad Jones performed the flow cytometry). Vesna Mihajlovic and Diana Hunter contributed to the qPCR in Figs. 3.3 and 3.7.
3.1 – Abstract:

Type 1 long-interspersed nuclear elements (LINE-1s) are autonomous retrotransposable elements that retain the potential for activity in the human genome. Several cases of genetic disease have been traced to gene disruptions caused by LINE-1 retrotransposition events in germ-line cells. The consequences of LINE-1 retrotransposition in somatic cells are less clear, however it has been proposed to contribute to oncogenesis and to cancer progression. LINE-1 retrotransposition may also play a physiological role in generating neuronal somatic mosaicism. In order to maintain genomic stability, the activity of these elements is suppressed by host-factors, including APOBEC3 proteins. We hypothesized that HIV-1 infection would compromise cellular control of LINE-1 elements, and result in the induction of retrotransposition events. Here we report that LINE-1 copy numbers, as measured by qPCR, increase dramatically in the DNA of HIV-1-infected primary T cells, with thousands of new copies per cell generated within 120 hours. We employed a targeted genome difference analysis (TGDA) technique to isolate new LINE-1 insertion sequences from the genomes of these infected cells. Using an eGFP-reporter based assay, we show that the HIV-1-Vif protein is necessary for the induction of LINE-1 retrotransposition. These data indicate that HIV-1 infection results in an extraordinary level of LINE-1 retrotransposition. This has allowed us to present the first demonstration of a novel endogenous LINE-1 element insertion in a primary human somatic cell. The de-repression of LINE-1 retrotransposition within HIV-1-infected cells allows for the direct assessment of the capacity for
LINE-1 replication within the human genome, revealing a surprising capacity for rapid induction in this context. These data further provide a novel mechanism for HIV-1-induced genomic instability and cell death, and have important implications for understanding interactions between exogenous retroviruses, endogenous retroelements and their hosts.

3.2 – Introduction:

LINE-1 element DNA sequences comprise approximately 17% of the human genome (17, 160). Although the bulk of these sequences are in the form of short 5’ truncated insertions, an estimated 100 full-length intact elements are present (40, 255). These intact LINE-1 elements represent the only retrotransposons encoded by the human genome known to be presently capable of autonomous replication (158, 208, 224, 255). Full-length LINE-1 elements are approximately 6 kb in length, comprising a 5’-untranslated region (UTR) two open reading frames (ORF1 and ORF2) and a 3’-UTR ending in a poly(A) tail (263). ORF1 encodes a 40 kDa protein with RNA chaperone activity, while ORF2 encodes a 150 kDa protein which possesses the reverse transcriptase (RT) and endonuclease functions required for retrotransposition (23, 84, 123, 152, 190-194, 208). Retrotransposition is thought to occur by a mechanism termed target-primed reverse transcription (TPRT), where reverse transcription is primed against genomic sequence at the insertion site, and thus occurs in concert with integration (61, 138, 276).
Several cases of genetic disease have been traced to gene disruptions caused by LINE-1 retrotransposition events in germ-line cells, and LINE-1 retrotransposition in somatic cells has been implicated in oncogenesis and cancer progression (141, 204, 213, 216, 245, 284). LINE-1 retrotransposition may also play a role in normal physiology. Previous studies have demonstrated the ability for tagged, engineered LINE-1 elements to retrotranspose in neural progenitor cells and this, supported by qPCR data showing elevated copy numbers of LINE-1 elements in the adult human brain, has led to the suggestion that LINE-1 retrotransposition may play a role in the generation of neuronal somatic mosaicism (62, 212). The vast amount of LINE-1 element sequence fixed in the human genome has, however, presented a technical challenge to the isolation of novel endogenous LINE-1 insertions in somatic cells.

The requirement for genomic stability in mammalian cells has resulted in the evolution of cellular factors responsible for restricting LINE-1 retrotransposition (52, 146, 210, 272). Some of these factors, such as members of the APOBEC3 family of proteins are also active against infectious retroviruses including HIV-1 (165, 184, 268, 269, 311). The HIV-1-Vif protein constitutes a viral countermeasure which induces the degradation of several APOBEC3 proteins, including 3F and 3G (187-189, 269, 273, 294, 307). We hypothesized that HIV-1 infection, through the actions of Vif, would compromise cellular control of LINE-1, resulting in the induction of retrotransposition events.
3.3 – Results:

3.3.1 – LINE-1 and Alu DNA copy numbers increase progressively in HIV-1-infected cells.

The outcome of a LINE-1 retrotransposition event is the generation of a novel copy of the element (often 5’ truncated) in the genome. We quantified LINE-1 DNA in primary CD4⁺ T cells that had either been infected in vitro for 168 hours with the HIV-1 molecular clone NL4-3, or had been treated as a mock-infection control. Two sets of LINE-1 primers were utilized in this experiment: one pair targeted the 3’ UTR of an alignment human specific (LINE-1Hs) and primate amplified (LINE-1PA) elements, while the second pair targeted ORF1 consensus sequence of highly active ‘hot’ LINE-1 elements (Brouha et al. 2003). In samples from three separate HIV-1-uninfected donors we observed elevated quantities of LINE-1 3’UTR and LINE-1-ORF1 DNA in HIV-1-infected cells as compared to mock-infected cells (LINE-1-ORF1 – mean 1.36 fold, \( p = 0.028 \); LINE-1-3’UTR – mean 1.52 fold, \( p = 0.006 \), Fig. 3.1A). The Alu elements are a class of non-autonomous retrotransposable elements that parasitize the LINE-1 retrotransposition machinery to achieve their own replication. We hypothesized that LINE-1 activity in HIV-1-infected cells would result in retrotransposition of Alu elements. We observed a 1.98 fold higher quantity of Alu DNA in HIV-1-NL4-3-infected cells as compared to mock-infected cells (\( p = 0.001 \), Fig. 3.1A). Similar results were obtained after infecting primary CD4⁺ T cells from tonsil-derived lymphoid tissue with HIV-1 (Fig. 3.1B).
In order to determine the kinetics of LINE-1 and Alu DNA increases in association with HIV-1 infection, we sampled mock and HIV-1-NL4-3 infected CD4+ T cells at 6 time-points. We observed progressive increases in LINE-1 and Alu relative DNA quantifications by SYBR qPCR, mirroring the kinetics of HIV-1-NL4-3 DNA replication (Fig. 3.1C-F). This progressive increase in LINE-1 and Alu quantification was observed in cells from 6 different HIV-1-uninfected subjects using a variety of experimental protocols. Results from a second experiment, using cells from a different individual, are shown in Fig. 3.1G. Increased LINE-1 copy number was also confirmed using Taqman qPCR with primers and probes for the TATA-box binding protein (TBP) genomic DNA sequence and for the consensus ‘hot’ sequence of LINE-1-ORF2. Absolute quantities at baseline and at multiple time-points post-infection were established by comparison to TBP and LINE-1-ORF2 containing plasmid standards, and are given in Fig. 3.1I. Representative absolute qPCR data is given in Fig. 3.1H.
Absolute quantitation of L1-ORF2 sequences at baseline and peak of HIV-1 infection.

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<th>Subject</th>
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Fig. 3.1. LINE-1 DNA copy number progressively increases in HIV-1-infected primary CD4+ T cells. (A) CD4+ T cells from three HIV-1-uninfected donors were either infected with 0.05 MOI of HIV-1-NL4-3 or maintained as mock infection controls. LINE-1-ORF1, LINE-1-3’UTR, Alu, Tim-3, and TBP were quantified in DNA from 168 hours post-infection using SYBR qPCR. In this experiment LINE-1-3’UTR was quantified using primers ‘LH1for/LH2rev’ (see Table 1). Shown are mean TBP-standardized quantitations in infected samples (black bars) expressed relative to the mock infection controls (white bars) for each donor. Six replicates were performed for each sample, error bars represent standard error, and p values were calculated by the Mann-Whitney test. (B) CD4+ T cells from tonsillar lymphoid tissue were either infected with 0.05 MOI HIV-1-NL4-3 or maintained as a mock infection control. Shown are DNA quantitations from 144 hours post-infection, standardized to the combined relative mean of 4 constant copy genes: NFib, β-actin, Tim-3, and TBP, and expressed relative to the mock-infection control. Three replicates were performed for each sample. (C-E) CD4+ T cells from an HIV-1-uninfected donor were either infected with 0.05 MOI of HIV-1-NL4-3 or maintained as a mock infected control. Samples were taken at indicated timepoints. (C) The kinetics of the HIV-1 infection were monitored at both the protein and DNA levels (note secondary y-axis). Samples were stained with fluoroChrome-conjugated antibodies to CD4 and HIV-1-Gag (intracellularly) and analyzed by flow cytometry. TBP, and HIV-1-gag were quantified in DNA from matched samples by SYBR qPCR. Shown are %Gag+ in NL4-3 infected (blue) and mock-infected (orange), as well as quantities of gag DNA in NL4-3-infected (red) and mock-infected samples (green). DNA quantitation was standardized to Tim-3, and expressed relative to a 144 hour NL4-3-infection DNA standard. (D-F) Shown are mean DNA quantitations for (D) LINE-1 3’UTR (using primers LH2for/LH3rev) (E) LINE-1-ORF1, and (F) Alu. In each graph, DNA quantifications for NL4-3 infected (solid lines) and mock infected (dashed lines) samples were standardized to Tim-3, and expressed relative to the baseline 0 hr sample. HIV-1-gag DNA quantification is overlaid as a reference (right y-axis). Six replicates were performed for every quantitation shown in D-F. P values were calculated by the Mann Whitney test, comparing mock-infected to HIV-1-infected at each timepoint * p < 0.05, ** p < 0.01, *** p < 0.001. (G) CD4+ T cells from a second representative HIV-1-uninfected donor were infected with 0.02 MOI of R5 tropic HIV-1-81A, or maintained as a mock infection control. LINE-1-ORF1, LINE-1-3’UTR (primers LH1for/LH2rev), Alu, Tim-3, β-Actin, TBP, and NFib DNA were quantitated in DNA from the indicated timepoints post-infection by SYBR qPCR. Shown are mean standardized DNA quantifications expressed relative to the mock infection DNA quantifications for each donor. Standardization was performed against a combined mean of the relative quantifications of the housekeeping genes: Tim-3, β-Actin, TBP, and NFib. Analogous experiments were performed on cells from 5 different donors with similar results. (H, I) CD4+ T cells from HIV-1-uninfected donors were either infected with 0.05 MOI of HIV-1-NL4-3 or maintained as mock infection controls. TBP and LINE-1-ORF2 were quantitated in DNA from the indicated timepoints using Taqman qPCR standardized to linearized plasmids. Six replicates were performed for each sample, and error bars in (H) represent standard error. (I) Shown are summary data indicating absolute LINE-1-ORF2 DNA copies/cell at baseline (pre-infection) and at the peak of HIV-1 infection (as determined by flow cytometry staining for HIV-1-Gag).

To determine whether LINE-1 copy number increases due to HIV-1 infection were restricted to HIV-1-infected cells, or were also associated with a bystander effect of infection, we sorted viable CD4+ T cell cells after 172 hours of HIV-1 infection with the R5 tropic strain HIV-1-81A into Gag+ and Gag− subsets by flow cytometry (Fig. 3.2A). We observed elevated levels of LINE-1-3’UTR and Alu in DNA from the Gag+ subset, but not in the Gag− subset, as compared to the mock infection control (Fig. 3.2B).
data support that HIV-1 infection results in the rapid amplification of LINE-1 and Alu DNA, consistent with the induction of LINE-1 retrotransposition events. Evidence supports that the primary mechanism of LINE-1 reverse transcription is target-primed reverse transcription (TPRT) (61, 138, 276), where reverse transcription is primed against genomic sequences and occurs concomitantly with integration. Thus, the increased LINE-1 DNA copy number in infected cells likely represents high-levels of de novo LINE-1 insertional events into genomic DNA (gDNA). However, these observations cannot strictly rule out that the increased LINE-1 DNA observed in these experiments was due to a novel mode of LINE-1 reverse transcription without integration. To test whether novel LINE-1 DNA sequences were integrated into high-molecular weight (hmw) gDNA, we obtained DNA from 168 hour HIV-1-NL4-3 and mock-infected CD4\(^+\) T cell samples and isolated hmw gDNA by electrophoresis (Fig. 3.2C). We observed significantly higher levels of LINE-1-3’UTR DNA in the gel purified hmw taken from HIV-1-infected cells as compared to that from the mock infected control (1.43 fold higher, p = 0.0018, Fig. 3.2D), supporting integration of novel LINE-1 DNA copies into genomic DNA.
**Figure 3.2.** LINE-1 copy number is increased in high molecular weight genomic DNA from HIV-1-infected primary CD4+ T cells. CD4+ T cells from HIV-1-uninfected donors were either infected with 0.05 MOI of HIV-1-81A or maintained as a mock infected control. (A) 172 hour post-infection cells were stained with the viability dye amine-red, and intracellularly with the Gag-specific antibody Kc57-Fitc. Viable cells were sorted into Gag− and Gag+ fractions based on the indicated gates. (B) DNA was isolated from sorted cells and quantification was performed by SYBR qPCR. Shown are absolute HIV-1-gag quantitations determined by comparison to HIV-1-gag and β-actin plasmid standards, as well as β-actin standardized LINE-1 3′UTR and Alu quantifications from sorted Gag+/Gag− populations expressed relative to a mock-infection control from the same donor. This experiment was performed for three donors with similar results. (C) DNA was isolated from cells 168 hours post-infection and ran on an agarose gel. High molecular weight (hmw) genomic DNA bands for mock and NL4-3 infections were purified by gel excision/extraction and concentrations were matched. (D) LINE-1-3′UTR, Tim-3, and TBP were quantified by SYBR real-time PCR. Shown are mean TBP-standardized quantifications of gel-extracted DNA expressed [from (C)] relative to the mock infection control. Tim-3 DNA was quantified as an additional validation of the PCR standardization and displayed no significant difference between mock and infected samples. Error bars represent standard error. P values were calculated by the Mann Whitney test * = p < 0.05, ** p < 0.01.
3.3.2 LINE-1 DNA copy number increase is preceded by a transient increase in LINE-1 RNA

The increase in LINE-1 DNA copy number was preceded by a transient increase in LINE-1 RNA in HIV-1-infected cells, however the latter did not show a progressive increase over-time, and therefore did not parallel the observed increases in LINE-1 DNA quantities (Fig. 3.3). Levels of LINE-1 RNA as measured by the LINE-1-3’UTR and LINE-1-ORF1 primers were elevated in HIV-1-infected primary CD4+ T cells at 24 hours post-infection (2.24 fold higher, p = 0.0049 for LINE-1-3’UTR, 1.97 fold higher, p = 0.0011 for LINE-1-ORF1), but neither LINE-1-ORF1 nor LINE-1-3’UTR RNA were significantly different from mock infection controls at 72 hours post-infection. At 144 hours infection, LINE-1-ORF1 RNA was marginally elevated in HIV-1-infected cells (1.22-fold, p = 0.0090) while the slightly higher level of LINE-1-3’UTR observed in HIV-1-infected cells did not reach statistical significance (1.23 fold higher, p = 0.1842). Although the fold differences in LINE-1 DNA and RNA quantitations, comparing mock and HIV-1-infected cells, were not strikingly different from each other, the fundamentally stable nature of genomic DNA as compared to the variable nature of RNA levels must be considered in interpreting these observations. The data therefore support a dramatic induction of LINE-1 retrotransposition in HIV-1-infected cells, in the context of only a modest effect on bulk LINE-1 RNA levels.
Fig. 3.3

Fig. 3.3. The effect of HIV-1 infection on LINE-1 transcript levels. Primary CD4+ T cells were isolated from an HIV-1-uninfected donor were stimulated in vitro for 48 hours and then either infected with 0.05 MOI of HIV-1-81A, or maintained as a mock infection control. Shown are RNA quantities of LINE-1-3’UTR (primers LH1for-LH2rev) and of LINE-1-ORF1 determined by SYBR qPCR, standardized to TBP, and expressed relative to a baseline (pre-infection, post-stimulation). At least 3 replicates were performed for each sample (means are shown), and the data are representative of 3 independent experiments. Error bars represent standard error.
3.3.3 – Novel LINE-1 insertions can be isolated from the genomic DNA of HIV-1-infected cells:

We applied a targeted genomic difference analysis (TGDA) technique to selectively isolate de novo LINE-1 insertions from the genomic DNA of HIV-1-infected cells. This method has been previously validated for describing distinct LINE-1 insertions between species, and is portrayed in Fig. 3.4 (45).
Fig. 3.4

**Fig. 3.4.** Schematic representation of Target Genome Difference Analysis technique (TGDA) used to identify novel LINE-1 inserts specific for HIV-infected cells. TGDA is based on specific PCR amplification of a group of genomic sequences of interest (in this case sequences flanking insertions of human LINE-1 retrotransposons amplified with primers specific to the retroelement 3′-terminus). These sequences are selectively PCR amplified and are subjected to subtractive hybridization, resulting in a library enriched in DNAs present in one of the comparing samples (tracer – DNA from infected cells) but absent from the other (driver – DNA from non-infected cells). The method includes two steps: whole-genome selective amplification of the sequence adjacent to interspersed repeats (in our case human LINE-1 retrotransposons) in both of the genomic DNA mixtures under comparison (A), and then subtractive hybridization of the selected amplicons (B). In the figure, orange and blue boxes denote infected-cell-
specific LINE-1 insertions and common LINE-1 insertions, respectively. In the first step (A) genomic DNA was digested with a frequent-cutter restriction enzyme at sites depicted by ‘R’ (A, stage 1, using Alu I). The resulting restriction fragments were then ligated to stem-loop structure-forming oligonucleotide adapters: A1A2 adapters for infected cell DNA and B1B2 adapters for uninfected cell DNA (A, stage 2). Yellow/red and light/dark green boxes designate suppression adapters used – with colors representing primer binding sites as follows: yellow – A1, red – A2, light green – B1, dark green – B2. As a result of adapter ligation, all DNA restriction fragments had inverted repeats at their termini. Therefore, the single-stranded fragments contained self-complementary termini capable of forming strong intramolecular stem-loop structures (panhandle-like structures). PCR amplification of the DNA fragments with such termini is suppressed when only one primer targeted at the 5’-ends of the ligated adapter is used (A, stage 2). In contrast, a pair of A1+LINE-1 or B1+LINE-1 primers (where LINE-1 are primers targeting the 3’ region of LINE-1 sequences) targeted at the single-stranded part of the stem-loop structure can initiate DNA synthesis by DNA polymerase (A, stage 2). The amplified DNA in this case will have different termini unable to form stem-loop structures, and can be further efficiently amplified. Thus, only regions containing 3’ LINE-1 DNA and a ligated adapter (A or B) were successfully amplified. Nested PCR with internal A2+L2 and B2+L2 primers was subsequently performed to increase the specificity of the amplification. Note that LINE-1 and L2 direct DNA synthesis towards the 3’ end of the LINE-1 element and into the flanking genomic DNA sequence (with the L2 binding site located 3’ of the LINE-1 binding site). This procedure therefore ensured efficient, nearly exclusive amplification of only the genomic DNA fragments that contained LINE-1 3’ terminal sequence and flanking genomic sequence (out to the point where the genomic DNA was digested and the adapter was ligated in the first step). The subtraction step is shown schematically on panel (B). Subtractive hybridization is based on re-association of DNA mixtures under comparison. The formation of a heteroduplex between tracer DNA with A1A2 adapters added to the 5’ of the sense strand (a subset of ‘portion A’) and complementary tracer DNA with A1 adapter added to the 5’ of the anti-sense strand (a subset of ‘portion B’) allows for subsequent amplification using an A1 primer. In contrast, formation of duplexes between tracer DNA (from either ‘portion A’ or ‘portion B’) with driver DNA results in a fragment which only contains A1 on one end, and therefore does not serve as a template for PCR amplification. After digestion and mixing at a large excess of one DNA (driver DNA = mock-infection DNA) over the other one (tracer DNA = HIV-1 infection DNA), the resulting short fragments were denatured and allowed to reanneal. During the re-association most of the tracer DNA hybridized to the excess driver DNA, except for the targets (sequences present in tracer, but not in driver DNA). The self-reassociated tracer, therefore, was enriched in LINE-1 insertion sites sequences that are unique to HIV-1 infected cell DNA fragments. We prepared two separate portions of the tracer DNA (B, left, stage 1) by re-amplification of the PCR products obtained at the previous stage (A, stage 3). We used the step-out PCR protocol with primers A1A2+A1+L2, or A1L2+A1+A2 for amplifications of portions A and B, respectively (Matz et al. 1999). The resulting ‘portion A’ DNA fragments contained A1A2 sequence at one end and L2 sequence at the other, whereas the corresponding terminal sequences of the portion B fragments were A2 and A1L2. To form 5’-protruding termini, we digested tracers A and B with the exonuclease Exo III until approximately 60 nt were removed from each 3’-end. These 5’-protruding single stranded termini (B, stage 2) prevented cross-hybridization of the repetitive LINE-1 sequences common to all of the amplicons, and therefore subsequent specific amplification of the double stranded tracer A/B heteroduplexes formed during the subtraction process. The driver DNA was digested similarly to remove ~40 terminal nucleotides. Tracers A and B and a 100-fold excess of driver (B, stage 3) were mixed, melted, and allowed to reanneal. The resulting mixture contained single stranded fragments of both tracers and of driver, double stranded hybrids formed between tracers and driver, homoduplexes formed due to self-reassociation of tracers A and B, and heteroduplexes formed by cross-reassociation of tracers A and B complementary strands (tracer A/B fraction). The protruding ends were then filled using DNA polymerase. The resulting heteroduplexes thereby acquired targets for primer A1 at both termini and were the only fragments that could be exponentially amplified with this primer (B, stage 3.3). The PCR products were cloned in plasmid vectors, transformed into e. coli and sequenced.
Putative de novo LINE-1 insertion sequences were identified in genomic DNA from infected CD4+ T cells, representing LINE-1 insertions into genomic sites that lacked any LINE-1 sequence in the human genome reference sequences. In order to confirm that these novel LINE-1 insertions were not part of the genome prior to HIV-1 infection, two sets of insertion site flanking primers were designed for each of these sequences: one against the human genomic reference sequence lacking the LINE-1 sequence, and the second spanning the insertional junction. A sequence was confirmed as a new insertion if it passed the following three criteria: i) the reference sequence lacking the LINE-1 sequence was successfully amplified from uninfected cell DNA from the same individual, ii) was shown to be homozygous by qPCR, and iii) the insertional junction sequence failed to amplify from uninfected cell DNA. Two sequences that passed each of these criteria are presented in Fig. 3.5. In each of these sequences, the LINE-1 element is separated from the flanking genomic DNA by a poly(A) sequence, this is consistent with the generation of this junction by a LINE-1 retrotransposition event.
Figure 3.5. New LINE-1 genomic insertions can be isolated from HIV-1-infected cells and sequenced. Shown are two LINE-1 genomic insertion site sequences isolated by the TGDA method (boxed), which were confirmed as being unique to infected cells by meeting the following criteria i) the reference sequence lacking the LINE-1 sequence was successfully amplified from uninfected cell DNA from the same individual and ii) was shown to be homozygous by qPCR iii) the insertional junction sequence failed to amplify from uninfected cell DNA (Fig. 3.6). LINE-1 sequences are indicated in red, while human genomic sequences are indicated in blue.
Data confirming the novelty of the insertion in sequence SH2_D012, by the criteria given above, is displayed in Fig. 3.6. Reference genomic sequence spanning the putative LINE-1 insertion site (without LINE-1 sequence) was successfully amplified from subject ‘OM406’ from whom the putative new insertion (SH2_D012) was identified, as well as from 6 control subjects (Fig. 3.6A). Primers spanning the putative novel insertional junction in SH2_D012 did not result in amplification of DNA from OM406 uninfected cell DNA, while successfully amplifying OM406 DNA which had been spiked with plasmid containing the SH2_D012 insertional junction sequence (Fig. 3.6B). Quantitative PCR showed very similar relative quantities of the reference genomic sequence (lacking LINE-1 insertion) in uninfected cells from OM406 as in two control subjects, supporting that these subjects are homozygous for the reference sequence (Fig. 3.6C). These data support that the SH2_D012 LINE-1 insertion isolated from HIV-1-infected OM406 cells was absent from OM406 cells pre-infection, confirming that the LINE-1 insertion sequence SH2_D012 represents a novel retrotransposition event. The TGDA method was also applied to two uninfected cell libraries from the same subject, and we were unable to isolate any sequences that passed the above criteria for novelty. This is consistent with the induction of LINE-1 retrotransposition in HIV-1-infected cells.
Figure 3.6. Confirmation that sequence SH2_D012 represents a de novo LINE-1 insertion. A. Primers were designed against the human genome reference sequence flanking the putative LINE-1 insertion site in the SH2_D012 sequence obtained from the TGDA analysis. If the template sequence contained a LINE-1 insertion then these primers would amplify a larger than expected band, or fail to amplify the target. Amplification of a product of the expected size from genomic DNA from this individual indicates the presence of the expected genomic sequence (without a LINE-1 insertion). B. Primers were designed that span the junctional sites of the putative new LINE-1 insertion SH2_D012. If the template sequence from these uninfected cells contained the putative de novo LINE-1 insertion then a product of the indicated mw would amplify. The SH2_D012 sequence was synthesized and cloned into plasmid pUC57 (Genscript) to serve as a positive control. Shown are the amplification products from uninfected cell DNA of the subject from whom the putative new insertion was isolated (OM406) – run in triplicate, as well as OM406 DNA spiked with the positive control plasmid – in duplicate, plasmid alone – in duplicate, and a non-template control (NTC). Products were separated through a 2.5% agarose gel, and the position of the expected mw product is indicated by an arrow. Note that 10ng of DNA were added to both D012-spiked and unspiked reactions. The greater abundance of higher molecular weight DNA in the latter is presumably due to amplification on non-specific template in the absence of the D012 template – for example, the LINE-1 primer acting as both forward and reverse primers for LINE-1 sequences inserted into the genome in opposite orientations. C. The primers targeting the human genomic reference sequences without LINE-1
insertions were used to quantify these sequences in DNA from uninfected OM406 cells as well as in DNA from control individuals (uninfected). TBP DNA was quantified in parallel and used to standardize the relative quantifications. The equivalent relative quantitation in these three samples is supportive of these individuals being homozygous for the expected genomic sequence (lacking the LINE-1 insertion).

3.3.4 – **Induction of LINE-1 retrotransposition in HIV-1-infected cells is observed using eGFP reporter retrotransposition assay:**

We corroborated these data using an *in vitro* retrotransposition assay, a modified version of a method that has been previously described (Fig. 3.7) (117, 208, 224). Jurkat cells were transfected with a plasmid containing the retrotransposition-competent human LINE-1 element LRE3 (99gfpLRE3) or a LINE-1 element that had been rendered retrotransposition incompetent by the introduction of point mutations in ORF1 (99gfpJM111). The LRE3 LINE-1 element was cloned from a patient with a case of chronic granulomatous disease that was caused by the retrotransposition of this element into the X-linked gene CYBB (201). The 3’ untranslated region of the LINE-1 element in these plasmids contains an eGFP cassette in inverse orientation, interrupted by an intron in the same transcriptional orientation as LINE-1. Expression of eGFP occurs only when the element has been transcribed, spliced, reverse transcribed and integrated into the genome (retrotransposed). Since Jurkat cells were only transfectable with these >18kb plasmids at fairly low frequencies, cells were selected with puromycin for 28 days prior to initiating assays. At this point we typically observed eGFP expression in 2-8% of 99gfpLRE3-transfected Jurkat cells, and an absence of eGFP expression in the 99gfpJM111 controls. These cells were then infected with HIV-1-NL4-3, or maintained as mock-infection control, and analyzed for eGFP and HIV-1-Gag expression by flow cytometry. Within infection cultures we considered bystander cells (Gag−) separately
from infected cells (Gag\(^+\)). We consistently observed that the percentage of eGFP\(^+\) cells increased progressively in Gag\(^+\) HIV-1-NL4-3-infected cells, peaking at 4 days post-infection (Fig. 3.7B).

To confirm that the increased level of eGFP observed in HIV-1-infected cells was indicative of an increased level of LINE-1 retrotransposition, we performed Taqman absolute qPCR of spliced eGFP and standardized this to the single-copy gene TBP (146). We observed 5-8 copies of spliced eGFP DNA (representing LINE-1 integration events) per cell at baseline in cells which had been stably transfected with 99gfpLRE3 for 28 days. This was in contrast to a lack of spliced eGFP in cells that had been stably transfected with the retrotransposition-incompetent 99gfpJM111 control (Fig. 3.7C). Levels of spliced eGFP in 99gfpLRE3 transfected cells increased progressively to a peak of 27.7±5.7 copies/cell by day 6 post-infection, concomitant with the peak of HIV-1 infection, and then began to decline. This closely paralleled the %eGFP\(^+\) cells as measured by flow cytometry which peaked at 6.3±0.2% at day 6 post-infection (Fig. 3.7C). These data support that the observed increase in eGFP expression frequencies in HIV-1-infected 99gfpLRE3 transfected cells are representative of the accumulation LINE-1 retrotransposition events. The frequencies of eGFP expression as measured by flow cytometry do, however, greatly underestimate true retrotransposition frequency, likely for the reasons that have been presented previously by others (224). For example, insertion of the eGFP cassette into a highly methylated region of the genome would result in little to no expression of eGFP protein. The gross underestimation of retrotransposition frequency by changes in eGFP frequency in the present system constitutes a likely
explanation for the seeming discrepancy in the scale of HIV-1-induced LINE-1 retrotransposition as determined by qPCR (Figs. 3.1, 3.2) versus that determined by eGFP expression (Fig. 3.7).

In order to determine whether HIV-1-Vif plays a role in the induction of L1 retrotransposition, we compared the effects of infection with wt and Vif-deleted (ΔVif) HIV-1-NL4-3 on eGFP-expression frequencies. Using a previously validated intracellular staining flow cytometric assay (289), we were able to readily detect APOBEC3G protein expression in uninfected Jurkat cells (Fig. 3.7D). This is consistent with our observations of severely impaired replication of HIV-1-NL4-3ΔVif in these cells as compared to wt HIV-1-NL4-3, indicating that these Jurkat cells are only semi-permissive to HIV-1-ΔVif replication (data not shown). Thus, the APOBEC3 system is at least partially intact in these cells and is vulnerable to impairment by Vif. Confirming this, infection of Jurkat cells with wt HIV-1-NL4-3, but not with HIV-1-NL4-3ΔVif, resulted in a reduction in APOBEC3G levels (Fig. 3.7D). A high-titer of both HIV-1-NL4-3 and HIV-1-NL4-3ΔVif were used in this, and in subsequent experiments, in order to obtain high levels of short-term infection despite the replicative deficiency of the ΔVif virus. Summary retrotransposition data from day 4 post-infection is shown in Fig. 3.7E with 36.0±1.2% of Gag+ cells expressing eGFP in the HIV-1-NL4-3 infections as compared to 14.0±0.5% of Gag− cells in the same sample. Substantially lower frequencies of eGFP+ cells were observed in HIV-1-NL4-3ΔVif infection cultures with 18.4±0.5% of Gag+ cells expressing eGFP versus 10.8±0.3% of Gag− cells (Fig. 3.7E, p<0.0001 for differences
between any two of the groups depicted). In a separate experiment, we infected Jurkat cells with twice the amount of ΔVif virus as wt virus (determined by p24 concentration), resulting in a higher level of infection with the former virus. Within the Gag\(^+\) population we continued to observe a markedly greater frequency of eGFP\(^+\) cells in wt as compared to ΔVif infections (Fig. 3.7F). Thus, HIV-1-induced L1 retrotransposition was predominantly limited to productively infected cells, with a substantially lesser bystander effect, and was largely dependent upon the presence of HIV-1-Vif protein.
**Figure 3.7.** HIV-1 infection enhances LINE-1 retrotransposition in Jurkat cells in a Vif-dependent manner. **A.** The plasmid 99gfpLRE3 encodes a retrotransposition competent LINE-1 element with an eGFP reporter cassette inserted into the 3’ UTR in reverse orientation. The eGFP coding sequence is disrupted by an intron in the same reading frame as the LINE-1 element. Thus, eGFP expression only occurs when LINE-1 has been spliced, reverse transcribed, and inserted into the genome. **B-F.** Jurkat cells were transfected with either the 99gfpLRE3 plasmid, or the 99gpJM111 retrotransposition incompetent control. These cells were selected with puromycin for 28 days, and then infected with either wt HIV-1-NL4-3 or HIV-1-NL4-3ΔVif. **B.** Shown are representative time-course data depicting % of cells...
expressing eGFP (x-axis) by days post-infection (y-axis). %eGFP in HIV-1-NL4-3-infected cells was determined by dividing the % of Gag+ cells expressing eGFP in infection cultures by the total %Gag+. %eGFP in bystander cells was determined by dividing the % of Gag+ cells in infection cultures expressing eGFP by the total % Gag+. C. HIV-1-NL4-3-infected Jurkat cells, stably transfected with either 99gfpLRE3 or 99gfpJM111, were collected at the indicated timepoints. One portion of these cells were analyzed for eGFP expression by flow cytometry, a second portion were lysed for DNA. Spliced eGFP DNA sequences were quantified by Taqman qPCR and standardized to TBP. Shown are qPCR and flow cytometry data from a representative timecourse experiment. Samples were run in triplicate for flow cytometry and in six replicates for qPCR, shown are mean values with error bars representing standard error. D. HIV-1-NL4-3 wt or HIV-1-NL4-3ΔVif infected Jurkat cells and mock infected controls were permeabilized and stained intracellularly with polyclonal anti-sera against APOBE3G followed by PE-conjugated secondary antibody. Shown are histograms of flow cytometry data depicting levels of APOBEC3G staining in each of these conditions, in comparison to staining with an isotype control. E. Shown are summary data from 4 days post-infection for uninfected cells, HIV-1-NL4-3 and HIV-1-NL4-3 ΔVif. Percentages of eGFP+ cells within infected (Gag+) and bystander (Gag-) cells were calculated and displayed separately. Replicates represent independent infections performed in parallel. P values for comparisons between any two of the depicted groups are <0.0001 by Student’s T test. F. Shown are flow cytometry plots of 4 days post-infection timepoints depicting eGFP on the x-axis (retrotransposition) and HIV-1-Gag on the y-axis. The data depicted in B, E and F are representative data from three separate experiments using separate batches of stably transfected Jurkat cells with varying frequencies of background eGFP expression. In total, the experiments depicted in B, E and F were repeated at least 10 times with similar results.

3.5 – Discussion:

These data provide evidence for an extraordinary level of LINE-1 retrotransposition in HIV-1-infected primary CD4+ T cells, with thousands of LINE-1 retrotransposition events per cell occurring within 120 hours of infection (Fig 3.1H), and a parallel amplification of Alu elements. The observation of retrotransposition on this scale is unprecedented in human cells, and provides a unique window into the intrinsic capacity for retrotransposition within our genomes and the mechanisms by which this is held in check. Our observation that the HIV-1-Vif protein was required for maximal induction of LINE-1 retrotransposition in primary cells, and was sufficient for induction of retrotransposition in Jurkat cells, support that the Vif-APOBEC3 interaction plays a primary role in this dysregulation, thus confirming earlier observations that APOBEC3 proteins play a role in the suppression of LINE-1 elements (52, 146, 210, 272). Although
Vif clearly plays a dominant role in HIV-1-induced LINE-1 retrotransposition, we did also observe that retrotransposition in cells infected with HIV-1 NL4-3ΔVif remained significantly elevated over mock-infection controls, leading us to speculate that HIV-1 may also interfere with additional LINE-1-suppressive factors, or may act to enhance or facilitate LINE-1 retrotransposition through, as yet, unknown mechanisms. The interaction of HIV-1 with endogenous LINE-1 elements thus provides a unique opportunity for future studies aimed at probing the mechanisms by which host cells suppresses LINE-1 retrotransposition, by first determining the means by which HIV-1 disrupts this control.

The rate of retrotransposition observed here in HIV-1-infected cells provides a potential mechanism for the previously observed, but poorly understood induction of genomic instability in HIV-1-infected cells where progressive aneuploidy has been described (195). The consequences of LINE-1 retrotransposition events include the generation of intrachromosomal deletions/duplications, interchromosomal translocations and the insertional disruption of genes (102). The scale of LINE-1 retrotransposition observed in HIV-1-infected cells thus indicates a surprising potential for the rapid destabilization of the human genome in the absence of sufficient control of LINE-1 elements. As this represents a major genotoxic stress, we speculate that the induction of LINE-1 retrotransposition constitutes a mechanism leading to the death of productively HIV-1-infected cells. This is consistent with previous studies which have demonstrated that the expression of HIV-1-Vif exerts a cytopathic effect in infected cells by causing cell cycle arrest at the DNA damage-induced G₂ checkpoint and that this, along with a
parallel effect induced by HIV-1-Vpr, is the predominant mechanism of HIV-1-induced cell death in vitro (252). In addition to a cytopathic effect, the mutational potential of active LINE-1 retrotransposition raises the potential that this phenomenon could contribute to the induction of malignancies in infected individuals, a possibility that will require further study.

A more thorough characterization of novel LINE-1 insertion sites and resultant mutations will help to address the overall contribution of HIV-1-induced LINE-1 retrotransposition to genomic destabilization. Our ability to isolate and characterize such insertions was hampered by the unique nature of each novel LINE-1 insertion in a population of HIV-1-infected cells. These HIV-1-infected primary cells are arrested in cell cycle and therefore do not divide to an appreciable degree. Hence, when a new LINE-1 insertion occurs in one of these cells, this insertion is not inherited by a pool of progeny cells, but rather remains unique. This provided a technical challenge in isolating single copies of novel LINE-1 insertions against a background of thousands of pre-existing genomic LINE-1 sequences which were shared by all cells from this subject. Furthermore, due to the unique nature of each sequence, once a LINE-1 insertion site template sequence was digested in the process of TGDA, this left no original template sequence on which to perform additional characterization. Future studies will address these issues through the development of novel techniques. Our findings demonstrate the largely unexplored potential for interactions between exogenous retroviruses, such as HIV-1, and endogenous retrotransposable elements. In order to fully understand the nature of the interaction between HIV-1 and a human cell it is clear that one must study
not only the interplay between HIV-1 and host genes, but also that between HIV-1 and the LINE-1 and Alu endogenous retrotransposable elements which together comprise some 27.5% of our genomes.

**Acknowledgments.** We thank John Moran and Jose Luis Garcia-Perez for providing the 99gfpLRE3 and 99gfpJM111 plasmids, as well as for helpful advice. We thank Robert Gifford and Neil Sheppard for helpful discussions. Biosafety level 3 laboratory space was provided by the Canadian Foundation for HIV Research (CANFAR) in partnership with the Ontario Innovation Trust and the Canadian Foundation for Innovation. The following reagents were obtained from the NIH AIDS Reagent Program: IL-2, pNL4-3, p197-1, and anti-hAPOBEC3G polyclonal antibody. MAO and DFN receive funding from Pfizer under a sponsored research agreement. This work was also supported by funds from the National Institutes of Health (AI076059 and AI084113), the J. David Gladstone Institutes, the Irvington Institute/Dana Foundation Fellowship from the Cancer Research Institute (to LCN), and the UCSF AIDS Research Institute (ARI). RBJ gratefully acknowledges scholarship support from the Ontario HIV Treatment Network (OHTN). MAO received salary support from the OHTN and the Canadian Institute of Health Research (CIHR). MAO also receives an unrestricted educational grant from Glaxo-Smith-Kline and Boeringer-Ingelheim. AAB was supported by the MCB program of the Russian Academy of Sciences, Russian Foundation for Basic Research and by the Russian President Grant Program. RBJ, KEG, DFN, MAO are listed as inventors on a patent application related to this work. The authors declare that they have no other competing financial interests.

**Author Contributions.** RBJ conceived the project. RBJ, KEG, HS, YX, AAB, NA, DVH, SM, VM, EM, DAM, LCN, DFN and MAO designed and performed experiments. DFN and MAO supervised the project. RBJ wrote the manuscript with input from all co-authors.
Chapter 4: Comprehensive cross-clade elimination of cells infected with globally diverse HIV-1, HIV-2 and SIV primary isolates by HERV-K(HML-2)-specific CD8+ T cells

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Contributions. Brad Jones was primarily responsible for producing the majority of these data (with technical assistance from co-op students). The following figures include data primarily generated by other researchers: Fig. 4.3 – Brad Jones infected these cells and shipped them to a team lead by Ingrid Pruimboom-Brees at Pfizer for immunohistochemistry analysis. Tables 4.1-4.3 – All work pertaining to T-cell receptor sequencing was performed by Eric Martin and Mark Brockman at Simon Fraser University.
4.1 – Abstract:

The genetic diversity of HIV-1 represents a paramount challenge in the development of a vaccine. Here, we establish rationale for a strategy which circumvents this obstacle by targeting a cellular immune response against a stable human endogenous retroviral (HERV) antigen as a marker of HIV-1-infected cells. HERV DNA sequences in the human genome represent the remnants of ancient retroviruses. We show that the infection of CD4$^+$ T cells with HIV-1 results in the induction of HERV-K(HML-2) transcription and protein expression. A HERV-K(HML-2)-Env specific CD8$^+$ T cell clone, obtained from an HIV-1-infected elite controller, responded to cells infected with HIV-1 in a Vif-dependent manner. Consistent with the proposed mode of action, this clone exhibited comprehensive elimination of cells infected with a panel of globally diverse HIV-1, HIV-2 and SIV isolates. The unprecedented breadth of reactivity provides impetus for exploring HERV-K(HML-2)-targeted HIV-1 vaccines and immunotherapeutics.

4.2 – Introduction:

The human genome contains an estimated 170 pro-viral copies of HERV-K(HML-2), some of which contain complete open reading frames for viral proteins (20, 26, 73, 115, 148, 179, 196, 211, 239, 280, 281, 301). Although no fully intact HERV-K(HML-2) pro-virus has yet been identified in the human genome, infectious HERV-K(HML-2) viruses can be reconstituted either from consensus sequences or by complementation between sequences from as few as three proviral loci (74, 167). Despite
this capacity for expression, HERV-K(HML-2) proteins have not been observed in healthy tissues, but rather have been uniquely associated with disease states such as teratocarcinoma (32, 118, 214, 248). We hypothesized that the manipulation of the host cellular environment by HIV-1, to one which favors retroviral expression and replication, may result in the expression of HERV-K(HML-2) proteins in infected cells. In support of this, we have previously reported that T cell responses to a variety of HERV-derived peptides, including a subset from HERV-K(HML-2), are detectable in HIV-1-infected subjects but not in uninfected controls (96). It has also been reported that HERV-K(HML-2) RNA is present at elevated levels in the blood plasma of HIV-1-infected subjects as compared to uninfected controls (58).

4.3 – Results:

4.3.1 – HIV-1 infection of primary CD4\(^+\) T cells results in the rapid accumulation of spliced and unspliced HERV-K(HML-2) transcripts.

We performed absolute quantitative RT-PCR on RNA from primary CD4\(^+\) T cells that had either been infected with the primary isolate of HIV-1 90TH_BK132 or maintained as mock-infected control. HERV-K(HML-2) gag, pol, and env probe/primers were designed to target uninterrupted viral sequences while the HERV-K(HML-2) rec probe/primers span the rec splicing site and therefore only amplify spliced viral RNA. We observed a rapid induction of HERV-K(HML-2) RNA as measured by each of these probe/primer combinations, the kinetics of which closely mirrored HIV-1 replication (Fig. 4.1A). HERV-K(HML-2) gag, pol and env templates were detected at similar
quantities to each other, consistent with the transcription of full-length HERV-K(HML-2) proviruses, and at their peak exceeded the levels of the TATA-box binding protein (TBP) transcript used to standardize the assay. Spliced HERV-K(HML-2)-rec RNA was present at very low levels in uninfected cells, but increased to approximately 10% the level of TBP at the peak of infection (Fig. 4.1A). This pattern of HERV-K(HML-2) induction was maintained when standardization was performed with a variety of housekeeping genes (Fig. 4.1B), and similar results were observed in cells from each of 3 other donors tested. We consistently observed a greatly diminished accumulation of HERV-K(HML-2) transcripts in cells infected with the lab-adapted isolate HIV-1-NL4-3, as compared to primary isolates, despite equivalent or greater levels of infection with the former. This is represented by spliced rec quantitation data from a second donor in Fig. 4.1C, but was observed for each of the HERV-K(HML-2) amplification targets (data not shown). Thus the infection of primary CD4+ T cells with primary isolates of HIV-1 results in the rapid accumulation of both spliced and unspliced HERV-K(HML-2) transcripts, with a substantially lesser effect observed upon infection with the commonly studied lab-adapted isolate NL4-3.
**Fig. 4.1**

(A) HERV-K-gag

(B) HERV-K-pol

(C) HERV-K-rec

Fig. 4.1. HIV-1 infection results in the accumulation of HERV-K(HML-2) RNA (A) CD4+ T cells were enriched from the PBMC of HIV-1-uninfected individuals, activated with anti-CD3/anti-CD28 and then either infected with primary isolates of HIV-1 or maintained as mock infection controls. Copy numbers of HERV-K(HML-2) and TBP RNA were determined using Taqman-based qPCR by comparison to serially diluted linearized plasmid standards. Shown are representative data from an infection with the HIV-1 isolate 90TH_BK132 depicting mean # HERV-K(HML-2) copies / copy TBP. Quantitations were performed in triplicate and error bars represent standard error. Also shown are levels of HIV-1 infection (% HIV-Gag+) at the indicated time-points as measured by flow cytometric staining for CD4 and HIV-Gag (right-panel). Similar data were observed in cells from each of four donors tested and with each of the two primary HIV-1 isolates tested (90TH_BK132 and 99UGA0848M1). (B) RNA from the sample analyzed in A was amplified using the MessageAmp kit (Ambion). HERV-K(HML-2)-pol RNA was quantified by Taqman qPCR and standardized to relative quantities of the housekeeping genes PP1A, GAPDH or β-Actin determined by SYBR qPCR. Values are expressed as Arbitrary Units = (standardized quantity) / (standardized quantity in uninfected 26 hour time-point). (C) Upper-panel: Shown are representative absolute qPCR data depicting copies of spliced HERV-K(HML-2)-rec / copy TBP for cells from a single subject infected with the indicated HIV-1 viruses. Quantitations were performed in triplicate and error bars represent standard error. Lower-panel: Shown are levels of HIV-1 infection corresponding to qPCR sampling points as measured by flow cytometry.
4.3.2 – HIV-1 infection of primary CD4+ T cells results in HERV-K(HML-2)-Gag and Env protein expression

We examined CD4+ T-cells which had been isolated from HIV-1-uninfected subjects and then either HIV-1-infected or mock-infected in vitro, for the expression of HERV-K(HML-2) Gag and Env proteins using commercially available antibodies. The anti-HERV-K-Gag mouse mAb HERM-1841 detected a 72 kDa band in HIV-1-infected cells which was absent from uninfected controls (Fig. 4.2A). This matched the migration of HERV-K(HML-2)-Gag detected in CD4+ T-cells transfected with the codon-optimized HERV-K(HML-2)-gag mRNA (Fig. 4.2A). The HERV-K(HML-2)-Gag protein was present at low abundance, with the image displayed in Fig. 4.2A representing an overnight exposure for HERV-K(HML-2)-Gag versus a 5 minute exposure for HIV-1-Gag. This was anticipated by our qPCR data where we typically observe HIV-1-gag RNA levels on the order of 2,500 copies/copy TBP versus <2 copies HERV-K(HML-2)-gag/copy TBP. The detection of HERV-K(HML-2)-Gag in these cultures was contingent upon high-levels of HIV-1-infection with a substantial proportion of late-stage infected cells (CD4 downregulated). Fig. 4.2B displays frequencies of HIV-1-infected (Gag+) cells in a subset of the samples lysed for the Fig. 4.2A western blot. The cells from subject OM620, in which HERV-K(HML-2)-Gag was clearly detected, were >61% infected, with 32.4% exhibiting late-stage infection. Cells from subject OM5037 with intermediate infections (90TH_BK132, 94US_33931N) gave rise to only faint bands whereas HERV-K(HML-2)-Gag was not detected in less advanced infections (00KE_KNH1135, 90US_873). The 98UG_57128 infection was unusual in that HERV-
K(HML-2)-Gag was detected despite very little HIV-1-Gag detection by western blot (Fig. 4.2A, 4.2B). By flow cytometry we observe that these cells were highly infected but that Gag staining was dim, suggesting that the epitope recognized by the anti-HIV-1-Gag mAb Ke57 may be mutated in this virus. We were able to enhance the sensitivity of HERV-K(HML-2)-Gag detection by immunoprecipitating (IP) HERV-K(HML-2)-Gag using the rabbit anti-HERV-K(HML-2)-Capsid pAb 4890-Gly and performing western blotting on the eluate with HERM-1841 (Fig. 4.2C). Thus, HIV-1-infection results in the induction of low-levels of HERV-K(HML-2)-Gag.
HIV-1 infection results in the induction of HERV-K(HML-2)-Gag protein expression. CD4$^+$ T-cells were enriched from the PBMC of HIV-1-uninfected individuals, activated with anti-CD3/anti-CD28 and then either infected with primary isolates of HIV-1 or maintained as mock infection controls. (A) Western blots of a single membrane probed consecutively with the anti-HERV-K(HML-2)-Gag mAb HERM-1841 (upper), the anti-HIV-1-Gag mAb Kc57 (middle) and an anti-Tubulin pAb. Samples represent infections and mock (uninfected) controls for two subjects: OM620 and OM5037. (B) Portions of the samples displayed in A were removed prior to lysis and stained with anti-CD4 (y-axis) and anti-HIV-1-Gag (x-axis). Shown are the resulting flow cytometry data. (C) Immunoprecipitations were performed on lysates from HIV-1-infected or uninfected primary CD4$^+$ T-cells using either an anti-HERV-K(HML-2)-Gag rabbit pAb or a rabbit IgG control. Immunoprecipitates were separated by SDS-PAGE and probed with the anti-HERV-K(HML-2) Gag murine mAb HERM-1841.

We have been unable to detect HERV-K(HML-2)-Env protein expression in HIV-1-infected cells by western blot using the mAb HERM-1811, while detecting expression in lysates of HERV-K(HML-2)-env mRNA transfected CD4$^+$ T-cells (data not shown). We have noted rapid degradation of these positive control samples, suggesting that the
transmembrane Env protein may be susceptible to aggregation following cell lysis. As such factors may have impaired our ability to detect low levels of Env expression by western blot, we also studied HERV-K(HML-2)-Env expression by immunohistochemistry. Fig. 4.3A-H depicts HIV-1-Gag staining in CD4+ T-cells from a single donor either maintained as a mock-infected control (A), infected with the lab-adapted HIV-1 isolate NL4-3 (B), or infected with one of the primary HIV-1 isolates 90TH_BK132 (C) or 99UG_A0848M1 (D). Fig. 4.3E-H depicts HERV-K(HML-2)-Env staining in matched samples using HERM-1811. In mock-infected cells (Fig. 4.3A and E), a nonspecific punctate cytoplasmic or membrane-associated staining (minimal in distribution and generally mild in intensity) was noted in a subset of mononuclear cells and cellular debris with both antibodies. We observed an induction of HERV-K(HML-2)-Env expression in cells infected with the primary isolates of HIV-1 90TH_BK132 (G) or 99UG_A0848M1 (H). In these samples, HERV-K(HML-2)-Env expression was induced on the membrane of many large mononuclear and syncytial cells (coarsely granular staining, up to marked intensity) and in the cytoplasm (homogenous staining, moderate intensity) of syncytial cells, mirroring the cell distribution and microscopic characteristics of the staining observed with the HIV-1-Gag antibody in the serial microscopic section. The specificity of the HERV-K(HML-2)-Env staining is supported by its agreement with our qPCR data in indicating greater induction of HERV-K(HML-2) in cells infected with primary isolates of HIV-1, as compared with cells infected with HIV-1-NL4-3, despite similar levels of infection (Fig. 4.3B and F). This demonstrates that HERM-1811 staining does not simply result from non-specific binding to large mononuclear and syncytial cells.
formed secondary to viral infection. Thus, HIV-1-infection results in expression of HERV-K(HML-2)-Env.

**Fig. 4.3**

**Fig. 4.3.** Expression of HERV-K(HML-2)-Env protein in HIV-1-infected primary CD4\(^+\) T cells. (A-D) Shown are immunohistochemistry data depicting primary CD4\(^+\) T cells stained with the HIV-1-Gag-specific antibody Kc57 of: (A) uninfected (B) HIV-1-NL4-3 infected (C) HIV-1-90TH_BK132 infected (D) 99UG_A0848M1 infected primary CD4\(^+\) T cells. (E-H) Shown are immunohistochemistry data from serial sections corresponding to those shown in (A-D) stained with the HERV-K(HML-2)-Env-specific antibody HERM-1811-5. Arrows emphasize examples of the staining characteristics described in the text.
4.3.3 – Isolation and characterization of a HERV-K(HML-2)-Env-specific CD8+ T cell clone from an HIV-1-infected elite controller:

The induction of HERV-K(HML-2) antigen expression in HIV-1-infected cells could serve to stimulate effector functions of HERV-K(HML-2)-specific CD8+ T cells, resulting in the elimination of HIV-1-infected cells. We examined this using a HERV-K(HML-2)-Env-specific CD8+ T cell response that we identified in the PBMC of an HIV-1-infected elite controller (subject ‘OM9’) using a peptide matrix mapping methodology in an IFN-γ ELISPOT assay (Fig. 4.4A). This response, which mapped to the 15mer peptide CIDSTFNWQHRILLV, was observed at a frequency of 138 sfu/10^6 PBMC at first sampling, approximately 11 years after infection, and increased progressively to 813 sfu/10^6 over the subsequent 5 years (Fig. 4.4B).
Fig. 4.4. Identification of a HERV-K(HML-2)-Env-specific CD8+ T cell response in PBMC from an HIV-1-infected elite controller. (A,B) Shown are IFN-γ ELISPOT results depicting mean spot forming units (SFU) /10^6 PBMC with error bars representing standard deviation. (A) All tests were performed in duplicate. (B) All tests were performed in quadruplicate.

CD8+ T cell clones specific for this peptide were obtained from this subject by an IFN-γ capture methodology. T cell determinant fine-mapping, performed on this clone (Fig. 4.5A) established CIDSTFNWQHR as the minimal determinant. HIV-1-Gag and CMV-pp65 specific CD8+ T cell clones were obtained from this subject in parallel to be used as controls in subsequent experiments.
Fig. 4.5

Fig. 4.5. Fine-mapping of the T cell determinant of the HERV-K(HML-2)-Env-specific CD8\(^+\) T cell clone. (A) The specificity of the HERV-K(HML-2)-Env-specific clone was confirmed using the original CIDSTFNWQHRILLV peptide (crude), a newly synthesized batch of the same peptide (>98% pure) and fine-mapped using a panel of truncated peptides. Shown are IFN-\(\gamma\) ELISPOT data depicting mean spots/10\(^6\) clone cells (tested in duplicate) with error bars representing standard deviation. (B, C) Shown are representative data from IFN-\(\gamma\) ELISPOT assays testing reactivity of the HERV-K(HML-2)-Env-specific T cell clone to serial dilutions of the indicated peptides in the presence of autologous B lymphoblastoid cell lines (BLCL). Tests were performed in triplicate and error bars represent standard error.
The T cell receptor sequences of the HERV-K(HML-2)-Env-specific T cell clone were obtained and are given below.

**Table 4.1. T cell receptor sequences.**

| Alpha | GAGCAGTGGATACACGCAGGATACATGGGGGAGCTGTGACGAGAGCAAGAGGTCA
|       | GAACACATCCAGACTCCTTAAGAGAA[A/G]CCTTTTGTGTTTGGAAACCTTTTCAAGG
|       | CCAGGGACTTGTCCAGCCCAACCTCCATTCTGCTAGCTCTCTGACTCGGACTGAGACCC
|       | CCTGCTCTGTCTCTCTCCCTGCTACGGTCTCAGGAGTTCTGCTAGCTCAGCCATGCT
|       | CGCTGCTCTGTCCAGGACTGAGGCTGAGTTTTTACTCTGGGAGGAACCAGACGACCC
|       | GAGAGGCTAATTGGTATAATTTTAAGAGAGTGAACCTC[CT][CT][CT][CT][CT]
|       | GAGAAGGACACTCACCACGACTCTTCTATATTCGACTGACACGAGTTCTCAGGCC
|       | TCAACACTCACTCTCCTCTATATACATCAACTACCTTCTCCTTGTGTAATGTGCAACACC
|       | AACGAGACAGTCAGCTGAGAAGACGACTCCCTCAACACACCCTACCCCTTGTTAAGAGC
|       | ATCCAGAGAATGTATGAGCCATATGAGCAGCCTGAGTACCTTGCTGCTGCTGCTGCTG
|       | CTCTGCTCTCTCTTTTTGTTGTTTGGAAACCTTTTCAAAGCCAGGGACTTGTCCAGCC
|       | CCATGCTCAGGAAGACCCCATATAGAGCAAGCGGCTGAGTACTTCTGCTGCTGCTGCT
|       | CTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG

| Beta  | GAGCAGTGGATACACGCAGGATACATGGGGGAGCTGTGACGAGAGCAAGAGGTCA
|       | GAACACATCCAGACTCCTTAAGAGAA[A/G]CCTTTTGTGTTTGGAAACCTTTTCAAGG
|       | CCAGGGACTTGTCCAGCCCAACCTCCATTCTGCTAGCTCTCTGACTCGGACTGAGACCC
|       | CCTGCTCTGTCTCTCTCCCTGCTACGGTCTCAGGAGTTCTGCTAGCTCAGCCATGCT
|       | CGCTGCTCTGTCCAGGACTGAGGCTGAGTTTTTACTCTGGGAGGAACCAGACGACCC
|       | GAGAGGCTAATTGGTATAATTTTAAGAGAGTGAACCTC[CT][CT][CT][CT][CT]
|       | GAGAAGGACACTCACCACGACTCTTCTATATTCGACTGACACGAGTTCTCAGGCC
|       | TCAACACTCACTCTCCTCTATATACATCAACTACCTTCTCCTTGTGTAATGTGCAACACC
|       | AACGAGACAGTCAGCTGAGAAGACGACTCCCTCAACACACCCTACCCCTTGTTAAGAGC
|       | ATCCAGAGAATGTATGAGCCATATGAGCAGCCTGAGTACCTTGCTGCTGCTGCTGCTG
|       | CTTCTGCTCTCTCTTTTTGTTGTTTGGAAACCTTTTCAAAGCCAGGGACTTGTCCAGCC
|       | CCATGCTCAGGAAGACCCCATATAGAGCAAGCGGCTGAGTACTTCTGCTGCTGCTGCT
|       | CTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG

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Table 4.2. Results summary from IMGT/V-QUEST tool – alpha chain.

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4.3.4 – Recognition of cells infected with diverse isolates of HIV-1 by HERV-K(HML-2)-Env-specific T cells:

The HERV-K(HML-2)-Env-specific CD8⁺ T cell clone was co-cultured for 6 hours with autologous primary CD4⁺ T cells that had either been infected with one of 3 diverse primary isolates of HIV-1 or maintained as an uninfected (mock) control. We observed striking induction of IFN-γ production and degranulation (CD107a) in clone cells that had been co-cultured with cells infected with each of the HIV-1 isolates (Fig. 4.6A). These responses were contrasted by a lack of recognition of uninfected autologous target cells, as well as of HIV-1-infected HLA-mismatched target cells, and were of similar magnitude to the response observed to uninfected cells pulsed with the cognate HERV-K(HML-2)-Env peptide (Fig. 4.6A). In parallel we tested the ability of HIV-1-Gag and CMV-pp65 specific CD8⁺ T cell clones to respond to HIV-1-infected and mock-infected target cells. As expected, we observed clear recognition of targets infected with clade B HIV-1 by the HIV-1-Gag-specific clone (Fig. 4.6B) and a lack of recognition with the CMV-pp65-specific clone (Fig. 4.6C). In agreement with our qPCR and immunohistochemistry data, showing only slight induction of HERV-K(HML-2) expression by HIV-1-NL4-3, we observed only marginal recognition of cells infected with this lab-adapted isolate by the HERV-K(HML-2)-specific clone despite high levels of infection (Fig. 4.6D, E). Further confirming the specificity of recognition, transfection of target cells with HERV-K-env-targeted siRNA prior to infection significantly impaired recognition by the HERV-K-Env-specific T cell clone, while having no effect on recognition by an HIV-1-Gag-specific T cell clone (Fig. 4.6F). Thus, the induction of
HERV-K(HML-2) antigen expression by HIV-1 results in the presentation of HERV-K(HML-2) derived peptides by infected cells and the activation of HERV-K(HML-2)-Env-specific T cells.
Fig. 4.6

HERV-K(HML-2)-Env-specific CD8+ T cells respond to cells infected with diverse primary isolates of HIV-1. (A-C) Shown are flow cytometry data, gated on CD8+ clone cells, depicting CD107a staining (degranulation) - y-axis by IFN-γ production - x-axis for (A) HERV-K(HML-2)-Env-specific (B) HIV-1-Gag-specific or (C) CMV-pp65-specific CD8+ T cell clones co-cultured with autologous CD4+ T cells that had either been infected with the indicated primary isolates of HIV-1, or maintained as uninfected (mock) controls. In A uninfected cells pulsed with the HERV-K-Env-derived CIDSTFNWQHRI peptide are shown as a confirmation of clone specificity. Also shown in A is a lack of recognition of HIV-1-
00UG_J3222M84-infected allogeneic CD4+ T cells (MHC-I-mismatched) by the HERV-K(HML-2)-specific T cell clone. (D) Data shown are analogous to those presented in A, and compare responsiveness of the HERV-K(HML-2)-Env-specific clone to cells infected with the lab-adapted isolate of HIV-1 NL4-3 with responsiveness to the primary isolate 90TH_BK132. (E) Shown are flow cytometry plots for CD4+ targets corresponding to D, representing the level of HIV-1 infection: HIV-1-Gag (x-axis), CD4 (y-axis). (F) Primary CD4+ T cells from subject OM9 were prepared for infection and then transfected with 165nMol of either one of two HERV-K(HML-2)-env-targeted siRNA duplexes or with a control non-targeting siRNA duplex. These cells were magnetofected with HIV-1 immediately after transfection and then used as targets in recognition assays 16 hours post-infection with either a HERV-K(HML-2)-Env or an HIV-1-Gag-specific CD8+ T cell clone. Relative recognition values were calculated as the ratio of: % CD107a+ (CD8+) in given condition / mean % CD107a+ (CD8+) in response to non-targeting siRNA controls. Shown are mean values from three replicates, with error bars representing standard error. P values were calculated by Student’s T test.

The recognition of HIV-1-infected cells by the HERV-K(HML-2)-Env-specific CD8+ clone was restricted by MHC-I as evidenced by the ability of the anti-MHC-I antibody DX17 to block recognition of HIV-1-infected target cells (Fig. 4.7).
Fig. 4.7

A

Mock (Uninfected)

HIV-infected + isotype control

HIV-infected + anti-MHC-I (DX17)

Mock + CI12 peptide + isotype control

Mock + CI12 peptide + anti-MHC-I (DX17)

B

\[ p < 0.0001 \]

\[ p < 0.0001 \]
Fig. 4.7. Blockade of HERV-K(HML-2)-Env-specific CD8$^+$ T cell clone recognition by anti-MHC-I antibody. CD4$^+$ T cells from subject OM9 were either infected with the HIV-1 primary isolate 90TH_BK132 or maintained as a mock infection control. These target cells were incubated with 10µg/ml of either the anti-MHC-I antibody DX17 or an isotype control. BLCL autologous to the HERV-K(HML-2)-Env-specific T cell clone were pulsed with CIDSTFNWQHRI peptide in the presence of 10µg/ml of either the anti-MHC-I antibody DX17 or an isotype control. These target cells (either CD4$^+$ T cells or BLCL) were then co-cultured with the HERV-K(HML-2)-Env-specific T cell clone. (A) Shown are representative flow cytometry plots depicting CD107a staining (degranulation) by IFN-γ. (B) Shown are summary data from 3 independent replicates.
To begin to probe the mechanism of HERV-K(HML-2) induction by HIV-1 we tested the effect of separately blocking HIV-1 entry, reverse transcription and integration with antiretroviral drugs (ARV) on the induction of recognition by the HERV-K(HML-2)-Env-specific T cell clone. A high-titer of HIV-1 was used, and recognition was tested 16 hours post-infection, to ensure that any observed effect could be attributed to blocking the specific replication phase of the inoculum virus. Each of the drugs tested effectively abolished recognition of target cells (Fig. 4.8A). Thus the completion of the infection cycle and integration of the HIV-1 provirus is necessary to induce HERV-K(HML-2) expression.
Fig. 4.8

**Fig. 4.8. Recognition of infected cells by HERV-K(HML-2)-Env-specific T cells is dependent upon completion of viral integration.** Primary CD4+ T cells from subject OM9 were treated with 1µM each of either efavirenz, nevirapine, integrase inhibitor 118-D-24, with 10µg/ml of T20, or with a combination of each of these drugs (ARV Mix) throughout a 16 hour exposure to the HIV-1 primary isolate 90TH_BK132. These target cells were then used in a standard recognition assay with the HERV-K(HML-2)-Env-specific CD8+ T cell clone. Responsiveness of this clone to drug-treated target cells was compared to mock-infected controls and to untreated cells infected with 90TH_BK132 in parallel. Shown are representations of flow cytometry data comparing the frequencies of a CD107aIFN-γ dual-positive clone cell populations in response to these target cells (black bars, left-axis). The degrees of HIV-1 infection in target cell populations were assessed by intracellular staining with the anti-HIV-1-Gag antibody KC57 (checkered bars, right-axis).
4.3.5 – Recognition of HIV-1-infected cells by HERV-K(HML-2)-Env-specific T cells is dependent upon HIV-1-Vif:

One potential mechanism of HERV-K(HML-2) induction by HIV-1, would be by the inhibition APOBEC family proteins by the HIV-1 Vif protein. To address this, we tested the ability of the HERV-K(HML-2)-Env-specific T cell clone to respond to primary CD4+ T cells infected with a series of Vif, Vpr, and Vpu deleted mutant viruses. Although these experiments were performed on an NL4-3 background (where HERV-K(HML-2) induction is weak), we were able to consistently observe subtle recognition of wt, ΔVpu and ΔVpr viruses contrasted by a lack of recognition of ΔVif and ΔVifΔVpr mutants (Fig. 4.9).
Fig. 4.9

**Fig. 4.9.** HIV-1-Vif is required for induction of HERV-K(HML-2)-specific T cell recognition of infected cells, while HIV-1-Vpr and Vpu are dispensable. HIV-1 NL4-3 and deletion mutants were produced by transfection of corresponding plasmids into 293T cells. CD4+ T cells from OM9 were either infected with one of these viruses, or maintained as mock-infection controls and then co-cultured with either HERV-K(HML-2)- HIV-1-Gag or CMV-pp65-specific CD8+ T cell clones. Shown are flow cytometry data, gated on CD8+ clone cells depicting CD8 – x-axis by CD107a (degranulation) – y-axis. Where cell numbers were sufficient, co-cultures were setup in duplicate (shown). The depicted results are representative of three independent experiments.
The dependence on Vif for the induction of HERV-K(HML-2) recognition was confirmed using wt and ΔVif variants of the primary isolate clone YU-2, where we observed clear recognition of wt virus and a lack of recognition of YU-2-ΔVif (Fig. 4.10A). In parallel, an HIV-1-Gag-specific CD8$^+$ T cell clone responded to cells infected with either YU-2 or YU-2-ΔVif (Fig. 4.10A). We tested the ability of the HERV-K(HML-2)-Env-specific T cell clone to respond to primary CD4$^+$ T cells which had been transfected with codon-optimized vif mRNA corresponding to the primary clade A isolate ELI, and observed a lack of recognition, despite Vif expression levels, and corresponding APOBEC3G knockdown, that were at least equivalent to that observed in HIV-1-infected cells (Fig 4.10B, C). Thus the expression of HIV-1-Vif is necessary, but not sufficient, for the induction of HERV-K(HML-2) expression.
Fig. 4.10. HIV-1-Vif is necessary, but insufficient, for induction of HERV-K(HML-2)-Env-specific T cell recognition. (A) Activated CD4⁺ T cells from subject OM9 were infected with HIV-1 YU-2 or YU-2ΔVif for 16 hours (both viruses produced by transfection of 293T cells with corresponding plasmids), or were maintained as mock infection controls. Target cells were then co-cultured with either the HERV-K(HML-2)-Env-specific or an HIV-1-Gag-specific CD8⁺ T cells clone. Shown are summary data from 4 independent replicates depicting frequencies of CD107α⁺ clone cells. P values were calculated using Student’s t-test. (B, C) Activated CD4⁺ T cells from subject OM9 were transfected with mRNA encoding eGFP, HIV-1-Gag, HIV-1-Vif (from primary isolate ELI) or mock-transfected (no RNA) and then co-cultured with HIV-1-Gag-specific or HERV-K(HML-2)-specific T cell clones in a standard recognition assay. (B) Shown are flow cytometry data from a representative experiment, gated on CD8⁺ clone cells and displaying IFN-γ (x-axis) by CD107α (y-axis). (C) Shown is a western blot depicting levels of APOBEC3G (upper) and HIV-1-Vif expression (middle) vs a Tubulin loading control (lower) in CD4⁺ T cells that had either been infected with HIV-1-90THBK132, maintained as an uninfected control, mock electroporated (no RNA) or electroporated with vif mRNA. Samples were run at 1x, 5x and 25x dilutions as indicated.
4.3.6 – Elimination of cells infected with diverse isolates of HIV-1, HIV-2 and SIV by HERV-K(HML-2)-Env-specific T cells, and suppression of viral replication:

To examine whether the recognition of HIV-1-infected cells by the HERV-K(HML-2)-Env-specific clone would result in the suppression of viral replication, HERV-K(HML-2)-Env, CMV-pp65, and HIV-1-Gag-specific CD8⁺ T-cells were co-cultured with autologous CD4⁺ T-cells that had been infected with HIV-1-91_US4 (Fig. 6A). Nine days post-infection we observed potent suppression of viral replication (p24 production) by both the HIV-1-Gag and the HERV-K(HML-2)-Env specific clones, and a lack of suppression by the CMV-pp65-specific clone. Since the HERV-K(HML-2)-Env-specific clone recognizes a stable host-genome encoded antigen, we reasoned that it should eliminate HIV-1-infected cells irrespective of HIV-1 sequence variability. To test this, we obtained a panel of 21 diverse HIV-1 primary isolates representing clades A-D, G and circulating recombinant forms (Supplementary Table 4) (41). CD4⁺ T-cells from OM9 were infected individually with viruses from this HIV-1 panel, as well as with HIV-2 and SIVmac isolates. Target cells were then either co-cultured with HERV-K(HML-2)-Env, CMV-pp65 specific CD8⁺ T-cell clones or maintained as ‘no clone’ controls. The HERV-K(HML-2)-Env-specific clone exhibited killing of cells infected with diverse isolates HIV-1, HIV-2 and SIV viruses tested, with statistically significant reductions in cells infected with each isolate except for 99UG_A0848M1 (Fig. 6B, C). In contrast, an HIV-1-Gag-specific clone, tested in parallel, failed to eliminate cells infected with HIV-2 and SIV viruses (Fig. 6D, and data not shown). This corresponds with mismatches between the HIV-1-Gag T-cell determinant recognized by the clone and the corresponding HIV-2
and SIVmac239 sequences (SIVmac239 is a cloned virus derived from the SIVmac251 swarm) (Fig. 6D). In comparing the HIV-2 and SIV elimination data to HIV-1 elimination data it is important to note that the anti-HIV-1-Gag antibody used for flow cytometry does not bind as well to HIV-2 or SIV Gag. Our flow cytometry substantially underestimates the amount of HIV-2 and SIV infection and hence the degree of elimination. Thus, consistent with the proposed mode of action, the HERV-K(HML-2)-Env-specific T-cell clone eliminated cells infected with diverse primate lentiviruses. We did, however, observe some heterogeneity in the ability of the HERV-K(HML-2)-Env-specific clone to eliminate cells infected with different isolates of HIV-1. On one end of the spectrum, cells infected with the clade B virus 94US_33931N or the clade D virus 00UG_J32228M4 were eliminated very effectively while, on the other end, we observed only a small degree of elimination of cells infected with the clade C virus 98US_MSC5016. We calculated an elimination ratio (% infected – no clone / % infected – HERV-K(HML-2)-Env-spec clone) and grouped viruses by clade (Fig. 6E). We observed a trend towards less effective elimination of clade C viruses as compared to viruses of other clades, however none of the differences between any two clades were significant (Fig. 6E). We did not observe a statistically significant reduction in cells infected with HIV-1 99UG_A0848M1 in our initial experiment. We posited that this was an experimental artifact, rather than a true inability of the HERV-K(HML-2)-Env-specific clone to kill cells infected with this isolate. In our initial experiment, the infection with 99UG_A0848M1 was particularly potent, such that the majority of cells were immediately infected by our synchronized magnetofection protocol. Thus at the
initiation of co-culture with clone, these cells had already been infected for 24 hours. Over the subsequent 24 hour co-culture period, the majority of these infected cells died whether or not they were co-cultured with clone, and thus we were unable to distinguish a significant effect of clone killing. When we repeated this experiment, tailoring the kinetics of the assay to this viral stock, we observed clear, dose-dependent, elimination of 99UG_A0848M1-infected cells by the HERV-K(HML-2)-Env-specific clone (Fig. 6F). In parallel, we repeated the elimination assay with the 98US_MSC5016 to ensure that the relatively modest, though statistically significant, elimination displayed in Fig. 6C was reproducible (Fig. 6F).
Fig. 4.11. HERV-K(HML-2)-Env specific CD8⁺ T-cells eliminate cells infected with diverse isolates of HIV-1 and suppress viral replication. (A) Primary CD4⁺ T-cells from subject OM9 were activated and then infected with the HIV-1 primary isolate 91_US4. These target cells were co-cultured with either the HERV-K(HML-2)-Env, HIV-1-Gag, or CMV-pp65 clone at the indicated effector:target ratios for 9 days. Concentrations of p24 in supernatants were determined by ELISA. Shown are results from a representative experiment depicting mean p24 concentration calculated from 5 replicates with error bars representing standard error. (B, C) Primary CD4⁺ T-cells from subject OM9 were infected with a panel of HIV-1, HIV-2 or SIV isolates for 24 to 72 hours. HERV-K(HML-2)-Env or CMV-pp65-specific T-cell clones were then added at a ratio of 1:10 effector:target and co-cultured for 24 hours. Levels of infection were measured by flow cytometry. (B) Representative data depicting elimination of cells infected with the clade D primary isolate of HIV-1 00UG_J32228M4. (C) Summary data for the panel of HIV-1, HIV-2 and SIV isolates. Statistical significance was calculated by probability binning using the built-in Flowjo algorithm, comparing target cells co-cultured with the HERV-K(HML-2)-Env-specific clone with those cultured in the absence of clone. * = p < 0.05 (T(X) > 3.0). ** = p < 0.01 (T(X) > 4.0. (D) Representative data from a SIVmac251 elimination assay. The experimental setup and data presentation are analogous to B, but include some additional controls. An HIV-1-Gag-specific CD8⁺ T-cell clone is shown to fail to eliminate cells infected with SIVmac251 (upper-right panel). The sequence of the T-cell determinant for this HIV-1-Gag-specific T clone is given below (HIV-1 subtype B consensus sequence), and is aligned with the corresponding sequence from SIVmac251-Gag. Amino acid residues in the SIVmac251 sequence which do not match the HIV-1-Gag sequence are highlighted in red. The lower-left panel demonstrates the effect of blocking antigen presentation using 10μg/ml of the anti-MHC-I antibody DX17, on elimination. (E) Using the data presented in C we calculated Elimination Ratio = % infected - no clone / % infected - HERV-K(HML-2)-Env-spec clone. There was no statistically significant difference between any of the two groups by Student’s t-test. (F) Shown are data from an additional elimination assay, testing the ability of the HERV-K(HML-2)-Env-specific T-cell clone to eliminate cells infected with 99UG_A0848M1 and 98US_MSC5016. Each virus was tested in triplicate and error bars represent standard deviation. P values were calculated by Student’s T-test.

4.3.7 – The HERV-K(HML-2)-Env-specific CD8⁺ T cell clone is not cross-reactive with HIV-1-Vif

Given that HIV-1-Vif is required for recognition of HIV-1-infected cells by the HERV-K(HML-2)-Env-specific T cell clone, we felt it important to rule out that the clone may be cross-reactive against a Vif-derived T cell determinant. Our data indicating that the expression of high levels of Vif is insufficient to induce recognition does argue against such cross-reactivity (Fig. 4.10). However, to test this more stringently we screened the HERV-K(HML-2)-Env-specific T cell clone for responsiveness to CD4⁺ T cells pulsed individually with 25μg/ml of 15mers overlapping by 11 spanning Vif (cat# 6446) , and observed no responsiveness (Fig. 4.12).
Fig. 4.12. HERV-K(HML-2)-Env-specific CD8⁺ T cell clone is not cross-reactive with HIV-1-Vif-derived peptides. Primary CD4⁺ T cells from subject OM9 were combined with the HERV-K(HML-2)-Env-specific clone at a ratio of approximately 1:1. Peptides were added to a final concentration of 25µg/ml, and cells were cultured for 6 hours in the presence of brefeldin A. Responsiveness of the clone was assessed by intracellular cytokine staining flow cytometry. Shown are summary data depicting the % of cells staining IFN-γ within the CD8⁺ clone population. HERV-K(HML-2)-Env-C112 is the optimal T cell determinant for the clone with the amino acid sequence CIDSTFNWQHRI while HERV-K(HML-2)-Env-CV15 is the extended T cell determinant CIDSTFNWQHRILLV. Peptides 6018 – 6064 are consensus clade B HIV-1-Vif spanning 15mer peptides overlapping by 11 (NIH AIDS reagent program, cat #6446).
4.4 – Discussion:

Our studies demonstrate the first example of specific recognition and elimination of HIV-1-infected cells by a non-HIV-1-specific T cell. This bears an important consideration for studies aimed at studying the T cell response to HIV-1-infection, by demonstrating that even a comprehensive evaluation of HIV-1-specific responses may not capture the sum of all T cell responses directed against HIV-1-infected cells. The clinical relevance of HERV-K(HML-2)-specific T cell responses, including any potential role in the phenomenon of elite control, will be evaluated in future studies. The breadth of reactivity of HERV-K(HML-2)-specific T cell responses against diverse isolates, both implied by the proposed mode of action and observed in the current study, comprises an enticing advantage over HIV-1-specific T cell responses which could be exploited in the development of HERV-K(HML-2) targeted vaccines and immunotherapeutics. The potential for autoimmunity associated with targeting an endogenous viral antigen will be an important consideration in such efforts, however we do note the lack of any such indications in the clinical history of the subject presented in the current study. In moving towards exploring HERV-K(HML-2) targeted vaccines it will also be important to determine whether SIV infection of non-human primates results in induction of simian endogenous retrovirus (SERV) expression and thus could serve as a model.

The present study also carries broader implications for understanding the interaction between HIV-1 and a human cell – where interplay between HIV-1 and HERVs may play an underappreciated role. A strong precedent exists in mice for
mobilization of endogenous retroviruses by exogeneous retroviruses where infection with exogenous ectopic MuLV has been shown to result in the replication of defective endogenous polytopic retroviruses (83). This has led to the suggestion that periods of replication of endogenous retroviruses in the mouse have been initiated by infection with exogenous retroviruses capable of circumventing restriction factors in the mouse. Despite a lack of overt sequence homology between HIV-1 and HERV-K(HML-2) a similar scenario exists where the HIV-1-Vif protein is known to degrade APOBEC3 proteins which have been shown to restrict the replication of reconstituted HERV-K(HML-2). Our demonstration that Vif is required for the induction of HERV-K(HML-2) expression supports that this may be a relevant point of interaction between HIV-1 and HERV-K(HML-2), although the apparent insufficiency of Vif to induce HERV-K(HML-2) expression points to the involvement of other factors.

In this study we present multiple lines of evidence indicating that infection with the lab adapted isolate of HIV-1-NL4-3 results in a much lesser induction of HERV-K(HML-2) expression than diverse primary isolates of HIV-1. The reasons for this are presently unclear, however it is intriguing to note that a recent study has demonstrated that NL4-3-Vif, while effective in degrading APOBEC3G, does not effectively degrade other APOBEC3 proteins including APOBEC3F (188). Further study is required to determine whether the requirement for Vif in inducing HERV-K(HML-2) expression is indeed related to its role in APOBEC3 degradation and hence whether the impaired functionality of some lab adapted isolates in this regard may underlie our observation. It will also be interesting to determine whether passaging NL4-3 for several rounds on
primary CD4+ T cells may result in reversion to a virus which is a more potent inducer of HERV-K(HML-2) expression. We also noted that the recognition of cells infected with YU-2 virus produced by transfected 293T cells, while more pronounced than recognition of NL4-3-infected cells, is still significantly less than that observed with primary isolate viruses. YU-2 is a molecular clone of a primary isolate of HIV-1 that was obtained from uncultured tissue(173). These results may indicate that even a single round of passaging on cell lines (in this case by transfection of plasmid into 293T cells) may result in partial loss of some functionality that is required for the induction of HERV-K(HML-2) expression. While further study is clearly required in this area, it is advisable at present that studies related to the interaction between HIV-1 and HERV-K(HML-2) should focus on primary isolate HIV-1 viruses which have been cultured on primary CD4+ T cells.

Annex to Chapter 4: Recognition of HIV-1-infected macrophages by LINE-1-specific CD4+ T cells

Our observations, highlighted in Chapter 4, that a HERV-K(HML-2)-Env-specific T cell clone specifically responds to and eliminates cells infected with diverse isolates of HIV-1, HIV-2 and SIV provides a powerful precedent for targeting HERV-K(HML-2)-derived antigens as surrogate markers of HIV-1-infected cells. We are presently working towards determining whether LINE-1-specific T cells exert similar functionalities. At present, we have isolated one CD4+ T cell clone specific for the ‘NLTQSRSTTWKL’ determinant of LINE-1-ORF2p. In initial experiments, we tested the ability of this clone to respond to and eliminate autologous HIV-1-infected CD4+ T cells and observed a lack
of recognition, while CD4\(^+\) T cells pulsed with the NLTQSRSTTWKL were recognized in parallel (data not shown). Since then, data have come to light which indicate that, for as yet unknown reasons, some HIV-1-specific CD4\(^+\) T cells are unable to respond to HIV-1-infected CD4\(^+\) T cells, but rather only recognize HIV-1-infected macrophages (251). The mechanisms for this observation are currently unclear. We have therefore recently revisited these experiments, testing the ability of the LINE-1-ORF2p-specific T cell clone to respond to autologous monocyte-derived macrophages infected with the CCR5-tropic primary isolate of HIV-1 BaL. We observed clear recognition of HIV-1-infected target cells by the LINE-1-ORF2p-specific T cell clone. An HIV-1-Gag-specific T cell clone tested in parallel responded to infected target cells to a similar degree, while a CMV-pp65-specific CD4\(^+\) T cell clone showed no response (Fig. 4.13). We are currently working to extend this observation by determining whether this recognition results in elimination of HIV-1-infected macrophages, and determining whether recognition is dependent upon HIV-1-Vif.
Fig. 4.13

**Fig. 4.13.** Recognition of HIV-1-infected macrophages by LINE-1-ORF2p-specific CD4\(^+\) T cells. LINE-1-ORF2p, HIV-1-Gag, and CMV-pp65 specific CD4\(^+\) T cell clones were isolated from subject 'OM77'. These clones were co-cultured with HIV-1-infected or uninfected autologous macrophages. (A,B) Shown are flow cytometry data. Clone cells were labeled with CFSE prior to co-culture to allow their discrimination (x-axis). IFN-\(\gamma\) is displayed on the y-axis as a measure of responsiveness. (A) Shown are responses of the LINE-1-ORF2p-specific clone to its extended determinant ‘NLTQSRSTTWKLNNL’, as well as to its minimal determinant ‘NLTQRSSTTWKL’.

The clone was also tested against an HIV-1-Gag peptide pool as a negative control. (B) Shown are flow responses of the CMV-pp65 (upper panel), HIV-1-Gag (middle panel) and LINE-1-ORF2p (lower panel) clones to uninfected and HIV-1-infected macrophages.

**Acknowledgments.** Funding for this work was provided by Pfizer through a sponsored research agreement. This work was also supported by funds from the National Institutes of Health (AI076059 and AI084113). We thank Tania Watts for useful discussion. We thank Michael Malim for providing the pYU-2 and pYU-2ΔVif plasmids and Eli Gilboa...
for provision of the pGEM/4Z/A64 plasmid. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann - La Roche Inc., pNL4-3, primary isolate HIV-1 viruses (Supplementary Table 2), integrase Inhibitor (118-D-24), nevirapine, efavirenz, and peptide sets representing HIV-1: Vif, Vpr, Vpu, Tat, Rev, Nef, Pol, Gag, and Env. RBJ gratefully acknowledges salary support from the Ontario HIV Treatment Network (OHTN). Biosafety level 3 laboratory space was provided by the Canadian Foundation for HIV Research (CANFAR) in partnership with the Ontario Innovation Trust and the Canadian Foundation for Innovation. RBJ, KEG, DFN and MAO are listed as inventors on a patent application related to this work. The authors declare that they have no other competing financial interests.

Author Contributions. RBJ, KEG, DFN and MAO conceived of the project. RBJ, KEG, SM, VM, NA, DVH, EM, VMJ, WZ, NFF, GG, NS, IMP, DG, LC, CS, SJH, JCW, KC, ED, HS, YX, DS, RT, JBS and MAB designed and performed experiments. MR and SMK produced custom anti-HERV-K(HML-2)-Gag antibodies used in experiments. EB, and CK provided clinical samples. PTL, NC, RBJ, DFN and MAO supervised the project. RBJ wrote the manuscript with input from all co-authors.
Chapter 5: HERV-K(HML-2) and LINE-1 specific T cell responses are detected at low frequency in HIV-1-infected subjects using 15mer peptides

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Contributions. Brad Jones was primarily responsible for producing the all of these data (with technical assistance from co-op students).
Abstract.

We have previously reported the detection of T cell responses to diverse families of human endogenous retroviruses (HERV) in HIV-1-infected subjects using predicted optimal epitopes. In the present study, we employ pools of overlapping 15mer peptides spanning HERV-K(HML-2)-Gag and Env as well as LINE-1-ORF2p to map T cell responses to these specific antigens in a cohort of HIV-1-infected subjects. Detection of these responses was confounded by the presence of false-positives, 3 of which were mapped to an HIV-1-Gag peptide contaminant. In the 27 HIV-1-infected subjects studied, only 3 bona-fide HERV-K(HML-2)-specific CD8+ T cell responses and 1 LINE-1-ORF2p-specific CD4+ response were detected. Thus, HERV-K(HML-2) and LINE-1-ORF2p specific T cell responses are either rare in HIV-1-infected subjects, or are not amenable to study with pools of 15mer peptides.

We have postulated that the manipulation of an infected cell by HIV-1 to favour retroviral expression and replication may result in the expression of HERV and LINE-1 antigens. To our knowledge, no studies to date have considered T cell responses specific for LINE-1 antigens in either HIV-1 or any other condition. We previously screened for HERV-specific T cell responses in a broad sense testing for CD8+ T cell responses to peptides derived from diverse HERV families, selected for their predicted binding to common MHC-I molecules, and observed frequent responses in HIV-1-infected subjects but not in uninfected controls (96). We have since reported that the presence of strong
HERV-specific T cell responses are associated with control of HIV-1 in chronic infection (SenGupta et al, in revision at J. Virol).

In following up on this observation, we have decided to focus in on the HML-2 lineage of the HERV-K class II superfamily, while screening for LINE-1-ORF2p-specific T cell responses in parallel. Although the first members of the HERV-K(HML-2) lineage colonized the human genome 35 million years (Myr) ago, recent proliferation is evidenced by the presence of human-specific and polymorphic HERV-K(HML-2) insertions. These recently integrated HERV-K(HML-2) proviruses are comparatively intact and many contain complete open reading frames for viral proteins (20, 26, 73, 115, 148, 179, 196, 211, 239, 280, 281, 301). We have recently demonstrated that a HERV-K(HML-2)-Env-specific CD8$^+$ T cell clone specifically responds to, and eliminates, cells infected with diverse isolates of HIV-1 (Jones et al., submitted).

We evaluated a potential role for HERV-K(HML-2) and LINE-1-ORF2p-specific T cell responses in natural control of HIV-1 infection using a standard epitope mapping methodology. We manufactured 15mer peptides which span the HERV-K(HML-2) Gag (172 peptides), Env (164 peptides) and LINE-1-ORF2 (306 peptides) proteins with 11 amino acid overlaps. These peptides were arranged into matrix pools of 10-18 peptides each to allow for mapping of individual peptide responses, as has been previously described for other antigens (3, 24, 27, 78, 145, 220, 242). PBMC from 27 HIV-1-infected subjects, comprising 11 chronic progressors and 9 viral controllers and 3 subjects in acute/early infection (see (136) for definitions) as well as 6 uninfected controls were screened by IFN-γ ELISPOT with final concentrations of 10μg/ml/peptide.
T cell responses to these HERV-K(HML-2) and LINE-1-ORF2p derived peptide pools were infrequently observed and were confounded by the presence of false positive responses which proved irreproducible with newly synthesized peptides.

The inability of some T cell responses, identified with our original HERV-K(HML-2) or LINE-1-ORF2p derived peptides, to recognize newly synthesized batches of the same peptides was studied using a set of three CD8+ T cell responses observed in the HIV-1-infected chronic progressor ‘OM125’. These responses, directed against HERV-K-Env-SSVQSVNFVNDWQKN (pep124), HERV-K-Gag-GIGQNWSTRSQQALM (pep100), and HERV-K-Gag-YENANPECQSAIKPL (pep121) were clearly identified by matrix mapping using our pre-established criteria of spot-forming units (SFU) that were >3x greater than background and > 50 SFU/million PBMC after background subtraction (Fig. 5.1A). All three of these responses were subsequently confirmed at the level of the individual peptide implicated by the matrix mapping (Fig. 5.1B and data not shown).
Fig. 5.1

CD8⁺ T cell responses can be mapped using matrix pools of HERV-K(HML-2) Gag and Env derived peptides. (A, B) Peripheral blood mononuclear cells (PBMC) from the HIV-1-infected subject ‘OM125’ were screened by IFN-γ ELISPOT using peptide-matrix pools. Shown are results depicting mean spot forming units (SFU) / million PBMC. Tests were performed in duplicate and error bars represent standard deviation. Background levels were established by measuring responsiveness to 0.5% DMSO, and a pool of CMV-pp65-peptides was included as a positive control. The horizontal line depicts the cut-off of a positive response based on both: i) >3x background and ii) >50 SFU/million PBMC after background subtraction. (C) Shown are IFN-γ ELISPOT with 200,000 OM125 PBMC/well confirming the response to the HERV-K(HML-2)-Env pep124 15mer peptide common to matrix pools 7 and 23. The responses to these matrix pools are also shown in parallel. The label ‘HK’ is an abbreviation of HERV-K(HML-2).
CD8+ T cell clones specific for these epitopes were established by a previously described method of IFN-γ capture followed by two rounds of limiting dilution cloning (137). These clones failed to respond to newly synthesized batches of their corresponding 15mer peptides (Fig. 5.2A), as well as to truncated peptides designed to fine-map their optimal epitopes (data not shown). Serendipitously, our experimental setup was such that mismatched epitopes were employed as negative controls for clone responses. For example, the HERV-K(HML-2)-Gag-pep100-specific clone was tested against HERV-K(HML-2)-Env-pep124 and HERV-K(HML-2)-Gag-pep121. Surprisingly, we observed that each of these clones responded to all three of these original peptides, while failing to respond to newly synthesized versions (Fig. 5.2B). A CMV-pp65-specific CD8+ T cell clone, tested in parallel, did not respond to the original HERV-K(HML-2)-derived peptides, ruling out a non-specific effect. This suggested that all three clones may be specific for a single foreign peptide contaminant that was common to all three of these peptide preparations. We tested the possibility that this peptide may be derived from HIV-1, by testing the ability of these T cell clones to respond to HIV-1 peptide matrix pools (peptides obtained from NIH AIDS reagent program cat#: 8117-Gag and 6208-Pol) and observed clear recognition of HIV-1-Gag peptide pools 5, 20 and 21 (Fig. 5.2B). The two 15mer peptides mapped by these responses, AAEWDRHLHPVHAGPI and DRLHPVHAGPIAPGQ share the known HLA-B*35-restricted T cell determinant HPVHAGPI – corresponding with the B35+ status of the subject. The possibility that these ‘clones’ may be oligo-clonal with distinct subsets specific for the HERV-K(HML-2)-derived peptides and HPVHAGPI was ruled out by flow cytometry experiments.
indicating responsiveness of the entire population to the original HERK(HML-2) peptide preparations (Fig. 5.2C). Furthermore oligo-clonality would not explain the failure of the clones to respond to newly synthesized batches of peptide. Thus the CD8$^+$ T cell responses identified using our original HERV-K(HML-2) peptides in subject OM125 are, in fact, specific for a foreign contaminant in the peptide preparation. We can rule out that the contamination occurred in our hands based on two lines of evidence. First, peptides were pooled into matrix pools by the manufacturer. It was mapping, based on these pre-assembled matrix pools, which lead us to test corresponding individual peptides, at which levels these responses were confirmed. Secondly, the original peptides were shipped direct from the manufacturer in two separate aliquots to our labs in Toronto and San Francisco. The contaminant-specific T cell clones responded to the corresponding HERV-K(HML-2) peptide preparations at both sites, while failing to respond to newly synthesized peptides.
Fig. 5.2 CD8+ T cell clones identified by responses to HERV-K(HML-2)-Env-pep124, Gag-pep100 and Gag-pep121 are specific for an HIV-1-Gag peptide contaminant. (A) CD8+ T cell clones responsive to original batches of HERV-K(HML-2) peptides were obtained by IFN-γ capture, followed by two rounds of limiting dilution cloning. Shown are IFN-γ ELISPOT results indicating that these T cell clones, presumably of 3 separate specificities, in fact respond to all three original HERV-K(HML-2) peptides, while failing to respond to newly synthesized batches of peptides with the same sequences. A CMV-pp65-specific CD8+ T cell clone did not respond to the original HERV-K(HML-2)-specific peptides, ruling out a non-specific effect. All peptides were tested at 100µg/ml in 0.05% DMSO. The label ‘HK’ is an abbreviation of HERV-K(HML-2). (B) These CD8+ T cell clones were tested against HIV-1-derived peptide-matrix pools to test the hypothesis that they were specific for a contaminating HIV-1 peptide. All three clones responded to matrix pools 5, 20 and 21, mapping to the 15mers AAEWDRLHPVHAGPI and DRLHPVHAGPIAPGQ, shown are the results for the clone obtained using HERV-K(HML-2)-Gag-pep100. (C) The CD8+ T cell clone from B was mixed with autologous B lymphoblastoid cells and either pulsed with peptide or maintained as a no-peptide control. Shown are flow cytometry data, gated on CD8+ cells, and depicting CD107a (y-axis) by IFN-γ (x-axis).
Although we did not specifically identify contaminants for other CD8⁺ T cell responses to original HERV-K(HML-2) or LINE-1-ORF2p derived peptides we have proceeded in our analysis to consider only responses that were reproduced by two batches of peptide from separate manufacturers to be valid. This left only two subjects, both controllers, with detectable HERV-K(HML-2) or LINE-1-ORF2p specific responses. One subject, OM77, made weak, but reproducible, responses to one epitope each in Gag and Env: HERV-K(HML-2)-Gag-pep123 QSAIKPLKGKVPAGS – mean 60 SFU/million PBMC, and HERV-K(HML-2)-Env-pep7 HKMNKMVTSEEQMKL – mean 65 SFU/million PBMC as well as one response to LINE-1-ORF2p NLTQSRSTTWKLNNL – mean 410 SFU/million PBMC. A second subject, OM9, made a response to HERV-K(HML-2)-Env-104 CIDSTFNWQHRILLV – 813 SFU/million PBMC, which has been subsequently fine-mapped to CIDSTFNWQHRI (Jones et al., submitted).

The discrepancy between the infrequent detection of T cell responses in the present study and the frequent detection of HERV-specific T cell responses reported previously by us likely results from a number of factors. Critically, in previous studies we considered T cell responses to diverse families of HERV, with only a minority of peptides representing HERV-K(HML-2). Furthermore, of the 6 HERV-K-derived peptides studied by us previously, 4 were from Pol – an antigen which was not included in the present study. In addition, in previous studies we tested individual peptides predicted to be optimal epitopes in an HLA-matched manner. This is almost certainly a far more sensitive method for detecting CD8⁺ responses than the use of pools of 15mers –
especially in the present case where HERV-K(HML-2)-specific responses may be of lower frequency and/or avidity than T cell responses to exogenous viruses (24, 27, 177).

We propose that the contamination issues encountered in this study are not related to a poor-quality supplier, nor to our misfortune, but rather that this may be a factor for which the study of HERV-K(HML-2) and LINE-1 specific T cell responses is likely to be particularly prone. Here, we are looking for low frequency responses, which when detected require fairly high levels of peptide (Jones et al., submitted), in subjects infected with HIV-1, a pathogen for which synthetic peptides are produced at high levels in facilities around the world. Thus, in addition to iterating the need, highlighted by others (66, 68, 69), for caution in considering the possibility of foreign contaminant in peptide preparations, we emphasize a specific need to consider the possibility of HIV-1 peptide contaminants when screening HIV-1 infected subjects for responses to HERV-K(HML-2), LINE-1 and other antigens.

In conclusion, the low level of detection of HERV-K(HML-2) and LINE-1-ORF2p specific T cell responses by screening with pools of 15mer peptides, compounded by the propensity for false positive responses, renders this an inefficient method for efforts aimed at studying associations between the presence these responses and natural control of HIV-1 infection. Alternative approaches, including transfection of dendritic cells with HERV-K(HML-2) and LINE-1 antigen encoding mRNA, will be explored by future studies. Although we consider it likely that the lack of T cell responses in the present study is due to suboptimal screening methods, we also acknowledge the possibility that this may reflect a true lack of T cell responses for this lineage in natural
HIV-1 infection. Given our recent demonstration of anti-HIV-1 activity by both a HERV-K(HML-2)-specific CD8⁺ T cell clone and a LINE-1-ORF2p-specific CD4⁺ T cell clone, the finding that such responses are not commonly induced in natural infection would highlight the potential for intervention by HERV-K(HML-2) and/or LINE-1 targeted therapeutic vaccination strategies.

**Acknowledgments.** Funding for this work was provided by Pfizer through a sponsored research agreement. This work was also supported by funds from the National Institutes of Health (AI076059 and AI084113). The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately and peptide sets representing HIV-1: Pol and Gag. RBJ gratefully acknowledges salary support from the Ontario HIV Treatment Network (OHTN). Biosafety level 3 laboratory space was provided by the Canadian Foundation for HIV Research (CANFAR) in partnership with the Ontario Innovation Trust and the Canadian Foundation for Innovation.

**Author Contributions.** RBJ, VMJ, DVH, EM, SM, VM, GG, PB, NS, DS, and RT designed and performed experiments. EB and CK provided clinical samples. RBJ, DFN and MAO supervised the project. RBJ wrote the manuscript with input from all co-authors.
Chapter 6: Expansion of low frequency HERV-K(HML-2)-specific T cell responses from ex vivo PBMC using mRNA transfected dendritic cells

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Contributions. Brad Jones was primarily responsible for producing the all of these data (with technical assistance from co-op students).
6.1 - Abstract:

We have previously screened PBMC from a diverse cohort of HIV-1-infected and uninfected subjects for T cell responses to HERV-K(HML-2) antigens using 15mer peptides, and observed only very infrequent responses. Here, we present the optimization of a powerful platform which utilizes professional antigen presenting dendritic cells (DCs) to expand low frequency HERV-K(HML-2)-specific T cells from PBMC. We demonstrate that this method successfully confirms T cell responses that were previously detected by ELISPOT, and can reproducibly detect novel responses that were undetectable by ELISPOT. HERV-K(HML-2)-specific T cells expanded in this manner exhibit specific recognition of autologous CD4+ T cells that had either been transfected with their corresponding antigen or infected with HIV-1. This provides critical evidence that the observed cross-clade recognition and elimination of HIV-1-infected cells by a HERV-K(HML-2)-Env specific T cell presented in Chapter 4 is likely to be generalizable to other HERV-K(HML-2)-specific responses.

6.2 – Introduction:

Dendritic cells (DCs) are professional antigen presenting cells, with the ability both to prime naïve T cell responses and to induce vigorous expansion of antigen-experienced memory cell responses (see (16) for review). These capacities have been utilized in in vitro to expand low frequency T cell responses in order to generate pools of antigen-specific T cells for reinfusion into patients. To date, these approaches have been
pursued primarily as immunotherapies against: certain types of cancers (reviewed in (229)), EBV-driven lymphoproliferative diseases (54, 119), and late recurrences of CMV disease (203). In addition to these therapeutic uses, in vitro expansion of T cell responses has been utilized in order to facilitate the study of low frequency responses that are present only below the limits of detection of conventional assays (121, 163, 266, 285, 290). Of particular relevance, it has been recently demonstrated that the co-culture of PBMC from breast cancer patients with autologous DCs transfected with HERV-K-env encoding mRNA results in the expansion of HERV-K-Env-specific CD8+ T cells. The observation that PBMC from healthy controls, tested in parallel, exhibited no such expansion provides evidence for expansion of a pre-existing, low frequency, HERV-K-Env-specific T cell response in breast cancer patients rather than de novo priming in this in vitro system.

Given that we have observed only very infrequent HERV-K(HML-2)-specific T cell responses in PBMC from HIV-1-infected subjects, we sought to apply a DC-based in vitro expansion system to determine whether additional responses were present below the limits of detection of our assays. This series of experiments were setup with three distinct objectives. Firstly, to determine whether the degree of ex vivo expansion of T cells (measured by CFSE diminution) in response to HERV-K(HML-2) transfected DCs correlates with HIV-1 status and clinical parameters. Secondly, to obtain HERV-K(HML-2)-specific T cell lines and clones in order to expand upon the observations of specific recognition and elimination of HIV-1-infected cells described in Chapter 4. Finally, to fully characterize expanded T cell responses by fine-mapping T cell determinants,
identifying their HLA restrictions, and assessing functional avidities. This would allow for rational development of an optimal peptide-based screening method for future studies. For example, perhaps pools of 11mer peptides overlapping by 9 would succeed in identifying \textit{ex vivo} T cell responses where 15mers overlapping by 11 failed. Given the labour and resource intensiveness of the DC expansion methods, the identification of an effective peptide-based screening method would greatly facilitate future studies.

\textbf{6.3 – Results:}

\textbf{6.3.1 – Nuclofection of HERV-K(HML-2) mRNA into dendritic cells results in high levels of antigen expression.}

Given the difficulties we have encountered with low levels of HIV-1-derived peptide contaminants leading to false positive responses, we opted to employ an alternative method of antigen presentation for this study – comprising electroporating immature DCs with codon-optimized, 5’-capped, poly(A)-tailed mRNA coding for GFP, HERV-K(HML-2)-Env, Gag and Rec, and HIV-1-Gag. We found this mRNA transfection to be preferable to plasmid DNA transfection in terms of transfection efficiencies, levels of antigen expression and cell viability (Fig. 6.1A). Expression of GFP, HIV-1-Gag, and HERV-K(HML-2)-Env in DCs was confirmed using a combination of flow cytometric and western blotting analysis (Fig. 6.1A-C).
Fig. 6.1

Transfection of DCs results in antigen expression. Immature DCs from an HIV-1-uninfected subject were electroprated with either plasmid DNA or mRNA encoding the indicated antigens. These cells were then matured for 24 hours (see methods). (A) Left panels: Shown are flow cytometry data depicting 7AAD staining (viability) by side-scatter (SSC) for DCs that had been transfected with either mRNA (upper panel) or plasmid DNA (lower panel) encoding GFP. Right panels: Shown are CD86 by GFP expression for the population gated in the corresponding 7AAD/SSC plots. (B) Shown is a flow cytometry plot for DCs transfected with HIV-1-Gag encoding mRNA depicting CD86 by HIV-1-Gag staining. Gates for Gag staining were drawn based on a no-RNA transfected control (not shown). (C) Shown is a western blot depicting HERV-K(HML-2)-Env expression in DCs transfected with the corresponding mRNA.
6.3.2 – Upregulation of CD25 activation marker on T cells in response to mRNA transfected DCs.

In initial experiments, we monitored the activation of CD4$^+$ and CD8$^+$ T cell subsets by measuring upregulation of CD25 following 16 days of co-culture with transfected DCs. We initially validated this method by screening an HIV-1-uninfected subject ‘OM630’ in comparison to an HIV-1-infected elite controller with a known HERV-K(HML-2)-Env-specific T cell responses – subject ‘OM9’. We observed clear upregulation of CD25 on CD8$^+$ T cells from OM9 in response to HERV-K-Env transfected DCs - 23.1% CD25$^+$ vs 4.34% background in mock-transfected control (Fig. 6.2A). A weaker response to HIV-1-Gag was also observed in OM9 – 6.48% CD25$^+$. In contrast, no responses were observed in the HIV-1-uninfected subject OM630. (Fig. 6.2B). The upregulation of CD25 on CD8$^+$ T cells in OM9 in response to HIV-1-Gag and HERV-K-Env was accompanied by expansion of the CD8$^+$ compartment as evidenced by a skewing of the CD8:CD4 ratio from 0.68 in the mock-transfected control to 0.75 in response to HIV-1-Gag and 0.96 in response to HERV-K-Env.
**Fig. 6.2**

OM9 - HIV-infected elite controller (known HERV-K-Env responder)

OM630 - HIV-uninfected

**Fig. 6.2. Confirmation of known HERV-K(HML-2)-Env-specific T cell response using DC expansion platform.** PBMC from known HERV-K(HML-2)-Env responder OM9 (highlighted in Chapter 4) and from HIV-1-uninfected subject OM630 were co-cultured for 16 days with autologous DCs that had been transfected with mRNA encoding the indicated antigens. Shown are flow cytometry data gated on CD8$^+$ (upper panels) or CD4$^+$ (lower panels) T cell subsets depicting CD25 (y-axis) as a measure of T cell activation.

In order to expand upon this result, and to test the reproducibility of the assay, we then performed two completely independent consecutive DC expansions using cells from the elite controller subject ‘OM2’. This subject had been screened for responses to
HERV-K(HML-2)-Gag and Env by peptide-matrix ELISPOT and no responses were identified. We observed clear induction of CD25 expression in this subject upon coculture with HIV-1-gag transfected DCs - 42.7% CD25\(^+\) vs 9.9% background in mock-transfected control (Fig. 6.3A). We also observed induction of CD25 in response to HERV-K-gag-transfected DCs – 15.9% CD25\(^+\). These results were reproduced in the independent replicate performed one week later. Note that in the second replicate we used anti-CD25-APC, as opposed to anti-CD25-FITC in the first experiments. This leads to enhanced staining of CD25 expressing cells in the former – but similar measures of % CD25\(^+\) (Fig. 6.3).
OM2 - HIV-infected viremic controller (no confirmed HERV-K-spec responses detected by peptide matrix)

**Fig. 6.3. Reproducibility of DC expansion platform.** PBMC from subject OM2 were co-cultured with autologous DCs that had been transfected with mRNA encoding the indicated antigens. This experiment was setup in completely independent replicates, one week apart from each other. Shown are flow cytometry data gated on CD8⁺ cells depicting CD25 expression as a measure of T cell activation.
6.3.3 – Quantitation of proliferation of T cells in response to mRNA-transfected DCs by CFSE diminution.

While the upregulation of CD25 proved to be a useful measure for optimizing this assay, the transient nature of its expression does not lend itself well to quantitative applications. For example, at a given time-point CD25 expression may underestimate the frequency of responding cells as downregulation of previously activated cells may already have occurred. Thus, we modified our protocol to include carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of PBMC prior to co-culture with transfected DCs. CFSE is an intracellular dye that is progressively diluted upon sequential rounds of cell division. Hence monitoring CFSE diminution by flow cytometry is commonly applied to assess cell proliferation. In the HIV-1-infected chronic progressor ‘OM5028’ we observed clear upregulation of CD25 on a population of CD8\(^+\) T cells in response to HIV-1-gag transfected DCs. This CD25\(^+\) population was essentially superimposable with the CFSE\(\text{dim}\) cells – indicating that they had undergone proliferation (Fig. 6.4).
Fig. 6.4

Expansion of T cells in response to mRNA transfected DCs can be measured by CFSE diminution. PBMC from the HIV-1-infected chronic progressor OM5028 were labeled with CFSE then cocultured for 16 days with autologous DCs that had been transfected with mRNA encoding the indicated antigens. Shown are flow cytometry plots depicting CFSE (proliferation) – x-axis by CD25 (activation) – y-axis.
Similar results were obtained using PBMC from 5 different subjects. This validates our previous assumption that CD25 expression marks cells undergoing expansion in response to antigen, and allows CFSE diminution to be used as a quantitative measure of \textit{ex vivo} proliferation in response to DCs.

6.3.4 – Recognition of HIV-1-infected cells by dendritic cell expanded HERV-K(HML-2)-specific T cell lines

We next sought to confirm the antigen specificity of CD8$^+$ T cell populations expanded in response to transfected DCs, as well as to examine whether these HERV-K(HML-2)-specific T cell lines would specifically respond to HIV-1-infected cells. The HIV-1-Gag, HERV-K(HML-2)-Gag and HERV-K(HML-2)-Env-specific cell lines expanded from OM2 were co-cultured for 8 hours with autologous CD4$^+$ target cells that had either been: i) transfected with mRNA encoding the corresponding antigen ii) mock-transfected with no RNA iii) infected with the HIV-1 clade C primary isolate virus 00UG_J3222M84 or iv) maintained as a mock-infected control. We observed clear recognition of both mRNA transfected and HIV-1-infected target CD4$^+$ T cells as measured by CD107a staining (degranulation) and IFN-$\gamma$ production (Fig. 6.5). This was contrasted by a lack of recognition of both uninfected and mock-transfected target cells. Thus the HERV-K(HML-2) Gag and Env-specific T cell responses expanded by DCs from OM2 specifically recognize HIV-1-infected cells.
Fig. 6.5. DC-expanded HERV-K(HML-2)-specific cell lines respond to antigen-transfected and HIV-1-infected CD4+ T cells. PBMC from subject OM2 were co-cultured with autologous DCs that had been transfected with mRNA encoding the indicated antigens. These expanded cell lines were then co-cultured with autologous CD4+ T cells that had either been transfected with mRNA encoding their corresponding antigen, infected with the clade C HIV-1 primary isolate 00UG_J3222M84, or maintained as mock transfected or uninfected controls. Shown are flow cytometry data, gated in CD8+ cells, depicting CD107a (degranulation) – y-axis, by IFN-γ production x-axis.
6.4 - Discussion:

Through extensive optimization, we have successfully developed a platform for expanding low frequency HERV-K(HML-2)-specific T cell responses from PBMC. This will now be applied to a diverse cross-section of subjects comprising: 30 HIV-1-uninfected subjects, 20 HIV-1-infected elite controllers, 30 HIV-1-infected chronic progressors and 20 individuals with acute HIV-1-infection. The degree of CFSE diminution following 16 days of co-culture will be used as a primary readout and this will be tested for correlations with HIV-1 status and clinical parameters such as CD4 count and viral load. We will also aim to map the epitopes targeted by DC-expanded HERV-K(HML-2)-specific T cell lines and to study the requirements for detecting responses to these epitopes \textit{ex vivo}. If we are generally unable to observe these responses in \textit{ex vivo} PBMC using optimal epitopes then this will lead us to conclude that HERV-K(HML-2)-specific T cell responses are present at such low frequencies that they are not amenable to study using conventional assays (such as ELISPOT). If, however, we observe that such responses are not detectable \textit{ex vivo} using pools of 15mer peptides, but are detectable using individuals peptides, or peptides of shorter lengths, then we will determine optimal conditions which will allow future studies to directly detect \textit{ex vivo} responses. The optimization of such conditions will allow us to consider correlations between functionality, breadth and magnitude of \textit{ex vivo} responses with clinical parameters. In interpreting the results of our cross-sectional assessment of HERV-K(HML-2)-specific proliferative responses, we will also remain cogniscent of the possibility that DCs may
prime *de novo* HERV-K(HML-2)-specific T cell responses *in vitro*. At present, we do not feel that our system shows evidence for *de novo* priming as we have yet to observe the induction of HIV-1-Gag-specific T cell responses in any of the 16 HIV-1-uninfected subjects tested. We have also observed a lack of HERV-K(HML-2)-specific T cell responses in these uninfected individuals.

Our demonstration that HERV-K(HML-2) Gag and Env-specific T cell lines, expanded from the PBMC of an HIV-1-infected elite controllers, specifically recognize HIV-1-infected cells adds support to the idea that the functionality of the HERV-K(HML-2)-Env-specific T cell clone highlighted in Chapter 4 may be generalizable. These expanded cell lines have now been plated at limiting dilution to generate clones which will be thoroughly examined. In addition, we will continue to directly test DC-expanded HERV-K(HML-2)-specific cell lines for recognition of HIV-1-infected cells until this has been demonstrated for lines from 5 different subjects. As of yet, no HERV-K(HML-2)-specific T cell responses have been identified in HIV-1-uninfected subjects, however any such response would be prioritized for recognition assays. The demonstration of specific anti-HIV-1 activity by HERV-K(HML-2)-specific T cells generated independently of HIV-1 infection (in uninfected subjects) would provide a powerful validation of our vaccine model. Finally, we are working towards incorporating LINE-1-ORF2p and HERV-K-Pol antigens into our DC expansion platform protocol such that responses to these can also be considered.
Acknowledgments. Funding for this work was provided by Pfizer through a sponsored research agreement. This work was also supported by funds from the National Institutes of Health (AI076059 and AI084113). The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Nevirapine, 3TC, HIV-1 primary isolates. RBJ gratefully acknowledges salary support from the Ontario HIV Treatment Network (OHTN). Biosafety level 3 laboratory space was provided by the Canadian Foundation for HIV Research (CANFAR) in partnership with the Ontario Innovation Trust and the Canadian Foundation for Innovation.

Author Contributions. RBJ conceived of the project. RBJ, SM, VMJ, VM, NFF, and GG designed and performed experiments. EB and CK provided clinical samples. RBJ, DFN and MAO supervised the project. RBJ wrote the manuscript with input from all co-authors.
Chapter 7: Tim-3 Expression Defines a Novel Population of Dysfunctional T Cells with Highly Elevated Frequencies in Progressive HIV-1 Infection

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Contributions. Brad Jones was primarily responsible for producing the majority of these data (with technical assistance from co-op students). The following figures include data primarily generated by other researchers: Fig. 7.1 c, d, e were performed and analyzed by Lishomwa Ndhlovu and Jason Barbour at UCSF. Statistical analyses for Fig. 7.3 were performed by Jason Barbour. Fig. 7.5d, e, f and g were performed by Lishomwa Ndhlovu. The phenotyping displayed in Fig. 7.8
was performed by Lishomwa Ndlovu. The analysis of signaling displayed in Fig. 7.9 was performed by Marc Schweneker. The generation of Tim-3-producing 293T-cell lines as well as optimization and validation of production and purification methods was a collaborative effort by Brad Jones and Malathy Satkunarajah.
7.1 – Abstract:

Progressive loss of T cell functionality is a hallmark of chronic infection with human immunodeficiency virus 1 (HIV-1). We have identified a novel population of dysfunctional T cells in HIV-1 infection marked by surface expression of the glycoprotein Tim-3. The frequency of this population correlated positively with HIV-1 viral load and inversely with CD4+ T cell count. In progressive infection, Tim-3 expression was upregulated on HIV-1-specific CD8+ T cells. Tim-3 expressing T cells failed to produce cytokine or proliferate in response to antigen, and exhibited impaired Stat5, Erk1/2, and p38 signaling. Blocking the Tim-3 signaling pathway restored proliferation and enhanced cytokine production in HIV-1-specific T cells. Thus, Tim-3 represents a novel target for the therapeutic reversal of HIV-1 associated T cell dysfunction.

7.2 – Introduction:

It is clear from many studies that HIV-1- or SIV-specific CD8+ and CD4+ T cell responses have an important role in containing viral replication (202, 222, 237, 247, 261). However, in most cases cellular immunity to HIV-1 proves incapable of long-term control of viremia and, without antiretroviral therapy, progression to AIDS occurs. The failure of the host immune system to contain HIV-1 is related to the functional impairment of HIV-1-specific CD8+ and CD4+ T cells which accompanies progressive HIV-1 infection, a phenomenon referred to as T cell exhaustion (12, 14, 38, 67, 120, 232, 234, 265, 267, 278, 279, 310). In HIV-1 infection, the deterioration of the T cell
response follows a characteristic pattern: proliferative capacity, cytotoxic potential, and the ability to produce IL-2 are lost early, while the production of IFN-γ is more enduring. Ultimately, the majority of T cells chronically exposed to HIV-1 antigens enter into a state of dysfunction and, as disease advances, even the ability to produce IFN-γ is progressively impaired (14, 71, 94, 155, 225, 246, 279). The causal relationship between this progressive T cell exhaustion and high levels of HIV-1 replication in progressive infection remains unclear. Recently, signaling through PD-1 was shown to play an important role in T cell exhaustion in three models of chronic viral infection: LCMV in mice, SIV in rhesus macaques, and HIV-1 in humans (19, 67, 90, 232, 233, 278). Blockade of the PD-1/PD-L1 signaling pathway results in enhanced T cell responses and viral control in murine LCMV infection, as well as in enhanced survival and proliferation of HIV-1-specific CD8+ T cells in vitro. Increased levels of total cytokine production, and increased frequencies of cells producing cytokine in response to antigen are also induced in 6 day in vitro cultures treated with anti-PD-L1 (12, 15). However, it has been demonstrated that there is no direct relationship between the level of PD-1 expression of an HIV-1- or SIV-specific CD8+ T cell and the ability of that cell to produce cytokine upon ex vivo stimulation (232, 233). This has led to the suggestion that the enhanced levels of total cytokine production observed in vitro with the addition of anti-PD-L1 is the result of greater survival and expansion of antigen-specific CD8+ T cells, rather than improved functionality on a per cell basis. These data suggest that PD-1 expression marks a population exhibiting features of relatively early T cell exhaustion, where cell survival and proliferation are impaired, but cytokine production remains intact. Thus the
mechanisms leading to advanced stages of T cell exhaustion, where cytokine production becomes impaired, remain largely undefined.

T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) is an immunoglobulin (Ig) superfamily member, that was identified as a specific cell surface marker of murine Th1 CD4+ T cells (206). Interaction of murine Tim-3 with its ligand galectin-9, regulates Th1 responses by promoting the death of IFN-γ producing Th1 cells (312). In mice, blocking the interaction of Tim-3 with its ligand(s) prevents the acquisition of transplantation tolerance induced by costimulatory blockade (254, 312). Furthermore, Tim-3-deficient mice are refractory to the induction of high-dose tolerance in an experimental autoimmune encephalomyelitis (EAE) model, and anti-Tim-3 monoclonal antibody treatment of SJL/J mice exacerbated EAE (206, 250). These results indicate that Tim-3 plays a role in suppressing Th1 mediated immune responses, at least partially through the termination of effector Th1 cells. In humans, a defect in upregulation of Tim-3 on IFN-γ producing CD4+ T cells has been implicated as a contributing factor to the pathology associated with multiple sclerosis (11, 149). No study has yet examined the role of Tim-3 in chronic viral infection.

7.3 – Results:

7.3.1 – Tim-3 expression on T cells correlates with clinical parameters of progression in HIV-1-infected individuals

We profiled Tim-3 expression by flow cytometry on PBMC from 9 HIV-1-uninfected individuals, and 31 treatment naïve, acute/early and chronically HIV-1-
infected subjects (CIRC cohort) that included both viral controllers (non-progressors) and progressors using a polyclonal anti-Tim-3 antibody. We observed elevated frequencies of Tim-3-expressing CD8$^+$ T cells in acute/early and chronic progressive HIV-1-infected individuals, but not in viral controllers, relative to uninfected individuals (28.5±6.8% for HIV-1-uninfected versus; 52.2±19.0% for acutely/early infected individuals, p=0.0015; 49.4±12.9% for chronic progressors, p=0.0003; and 31.6±7.3% for viral controllers, p=0.48) (Fig. 7.1a,b). Tim-3 expression was also elevated on CD4$^+$ T cells from acutely/early infected individuals and chronic progressors, as compared to both viral controllers and HIV-1-uninfected individuals (Fig. 7.1a,b). The frequency of Tim-3$^+$ CD8$^+$ T cells correlated positively with HIV-1 viral load (p < 0.0001, Fig. 7.1d), and inversely with absolute CD4$^+$ T cell counts (p < 0.0001, Fig. 7.1d). Similarly, the frequencies of Tim-3$^+$ CD4$^+$ T cells were significantly correlated with viral load (p=0.0087) and absolute CD4$^+$ T cell counts (p=0.0273) (Fig. 7.1d). T cell activation as reported by CD38 expression is an additional predictor of disease progression (103). CD38 expression on CD8$^+$ T cells correlated with the frequency of Tim-3$^+$ CD8$^+$ T cells (p < 0.0001, Fig. 7.1d), and CD38 expression on CD4$^+$ T cells correlated with the frequency of Tim-3$^+$ CD4$^+$ T cells (p < 0.05, Fig. 7.1d). In a separate cohort of 60 treatment naïve, acutely/early HIV-1-infected individuals (OPTIONS cohort), we observed an analogous increase in the frequency of Tim-3$^+$ CD8$^+$ and CD4$^+$ T cells as assessed with a monoclonal anti-Tim-3 antibody (Fig. 7.1c). Similar positive correlations between HIV-1 viremia, CD38 and Tim-3 expression on T cells were also observed in this acute/early infection cohort (Fig. 7.1e).
**Fig. 7.1**

**a** HIV-1 Uninfected  

**b** HIV-1 Infected  

**c** HIV Uninfected  

**d** HIV Infected  

**e** HIV Uninfected  

**f** HIV Infected  

**g** HIV Uninfected  

**h** HIV Infected  

**i** HIV Uninfected  

**j** HIV Infected  

**k** HIV Uninfected  

**l** HIV Infected  

**m** HIV Uninfected  

**n** HIV Infected  

**o** HIV Uninfected  

**p** HIV Infected  

**q** HIV Uninfected  

**r** HIV Infected  

**s** HIV Uninfected  

**t** HIV Infected  

**u** HIV Uninfected  

**v** HIV Infected  

**w** HIV Uninfected  

**x** HIV Infected  

**y** HIV Uninfected  

**z** HIV Infected  

**Fig. 7.1.** Tim-3 is upregulated on T cells in HIV-1 infection and its expression correlates with parameters of HIV-1 disease progression. (a) PBMCs from HIV-1-infected and uninfected subjects were stained with antibodies against CD4, CD8, CD3 and a biotinylated polyclonal goat anti-Tim-3 antibody, followed by a secondary streptavidin-APC conjugate. Plots show events gated on the CD3⁺ population, and subsequently on the CD8⁺ or CD4⁺ populations, from a representative HIV-1 uninfected subject and a chronically HIV-1 infected subject. Biotinylated normal goat serum was used as a negative control. (b, c) The percentages of Tim-3⁺ cells within CD8⁺ and CD4⁺ T cell populations are indicated for (b) 40 individuals from the CIRC cohort separated into the following groups: HIV-1 uninfected (9), acute/early HIV-1-infected (7), HIV-1-infected chronic progressors (16), and HIV-1-infected viral controller (8) using polyclonal goat anti-Tim-3 Ab, and (c) 60 treatment naive HIV-1 infected individuals from the UCSF OPTIONS of primary infection and 9 HIV-1 uninfected controls using PE conjugated monoclonal anti-Tim-3 Ab. Subjects from the CIRC cohort were defined as follows: acute/early = infected with HIV-1 < 4 months; chronic progressor = infected > 1 year with CD4⁺ T cell count decline >50 cells/mm³/year; viral controller = infected > 1 year, no evidence of CD4⁺ T cell count decline, and viral load <5,000 copies/ml bDNA. Characteristics of the OPTIONS acute/early infection cohort are detailed in methods. Statistical analyses for both cohorts were performed using the Mann-Whitney test. (d, e) Correlation between Tim-3
expression on CD8$^+$ and CD4$^+$ T cells and viral load, CD4$^+$ T cell counts, and levels of CD38 expression among individuals with available clinical data from the (d) CIRC cohort and (e) OPTIONS cohort. Shown for the CIRC cohort are Tim-3 levels determined using a polyclonal anti-Tim-3 antibody. For the OPTIONS cohort, levels of Tim-3 expression were assessed using the monoclonal anti-Tim-3 Ab. Both antibodies are from R&D systems. Statistical analyses were performed using the Spearman’s rank correlation test.

**7.3.2 - Tim-3 is upregulated on HIV-1-specific CD8$^+$ T cells in progressive HIV-1 infection**

Tim-3 expression on antigen specific CD8$^+$ T cells was examined in HLA-A*0201$^+$, HLA-B*0702$^+$, and HLA-B*0801$^+$ chronically HIV-1-infected individuals using matched MHC-I pentamers. We observed significantly higher levels of Tim-3 on HIV-1-specific versus CMV-specific CD8$^+$ T cells (p=0.0065 by MFI, p=0.0026 by %Tim-3$^+$, Fig. 7.2a-e). CMV-specific CD8$^+$ T cells exhibited low levels of Tim-3 expression, with the exception of one response to CMV-pp65-TPRVGGGAM, which exhibited high levels of Tim-3 expression as measured by MFI, observed in cells from an individual with AIDS (Abs CD4 count = 132 cells/µl). Tim-3 expression was heterogenous amongst HIV-1-specific responses with some exhibiting very high levels of Tim-3, while others exhibited only baseline levels (Fig. 7.2c,e). The heterogeneity observed in Tim-3 expression levels on HIV-1-specific CD8$^+$ T cells cannot be attributed solely to inter-subject variability, as responses with high levels of Tim-3 expression were frequently observed contemporaneously with responses exhibiting low levels of Tim-3 expression within the same individual. This leads us to speculate that Tim-3 expression may mark HIV-1-specific T cells with differing functional capacities.
**Fig. 7.2**

a) CMV-pp65 A2*NLVPVMATV

b) Tim-3 MFI

p = 0.0065

CMV-specific HIV-1-specific

CD8

Tim-3

CMV-specific HIV-1-specific

p = 0.0026
**Fig. 7.2. Tim-3 is Expressed at Elevated Levels on HIV-1-Specific CD8+ T Cells in Progressive HIV-1 Infection.** PBMC from HLA-A*0201+, HLA-B*0702+, and HLA-B*0801+ chronically HIV-1 infected individuals from the CIRC cohort were stained with matched HLA pentamers presenting CMV, EBV, influenza and HIV-1 epitopes, and with anti-Tim-3. Shown are (a) representative flow cytometry data from one HIV-1-infected chronic progressor using HLA-A*0201 pentamers presenting the CMV-pp65 epitope ‘NLVPMTATV’, the EBV-Bmlf1 epitope ‘GLCTLVAML’, the HIV-1-Pol epitope ‘ILKEPVHG’ and the HIV-1-Gag epitope ‘SLYNTVATL’. Compiled Tim-3 expression data from (n=41) chronic progressors is shown for (b,d) pooled Tim-3 expression on HIV-1 and CMV specific CD8+ T cell responses from chronic progressors (c,e) individual epitope responses. Statistical analyses comparing pooled responses were performed using the Mann-Whitney test.

### 7.3.3 - Reduction of Tim-3 expression upon initiation of HAART is correlated with levels of ongoing T cell activation (CD38 expression)

The effect of highly active antiretroviral therapy (HAART) on Tim-3 expression was studied in 7 chronically HIV-1-infected individuals at baseline and at 1, 2, 3 and 6 months post-initiation of HAART (Fig. 7.3). Four subjects with chronic infection demonstrated a steady decline in Tim-3 levels on both CD4+ and CD8+ T cells with HAART, while three subjects (OM 304, 331, 287) maintained high levels of Tim-3 expression despite achieving undetectable HIV-1 viral loads (<50 copies/ml bDNA) (Fig. 7.3a,b). In a mixed-effects longitudinal analysis we observed that CD8+ T cell activation as measured by CD38 expression was found to be significantly associated with Tim-3 expression over the period of HAART. Both the percentage of CD8+ T cells expressing CD38, and the CD38 median fluorescence intensity on CD8+ T cells each associated with higher Tim-3 percentages on CD8+ T cells during therapy (0.38 (SE =0.11) percentage point higher Tim-3 expression on CD8+ per each 1 percent higher CD38 expression on CD8+ T cells (p=0.001; Fig. 7.3b), and 0.7 (SE=0.19) percentage point higher Tim-3 expression on CD8+ per each 1 unit higher CD38 MFI on CD8+ T cells (p=0.001). These effects remained unaltered when adjusted for CD4+ T cell count. In contrast, neither HIV-1 viral load (p=0.25) nor absolute CD4+ T cell count (p=0.07), were significantly
associated with Tim-3 expression post-HAART. Maintenance of high levels of Tim-3 expression in a subset of chronically HIV-1-infected individuals treated with HAART therapy is thus related to ongoing T cell activation (CD38 expression).

**Fig. 7.3**

<table>
<thead>
<tr>
<th>Associations with Tim-3 expression on HAART</th>
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<tbody>
<tr>
<td>Viral Load (copies/ml)</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>CD8 + Tim-3+</td>
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</table>
Fig. 7.3. Effect of HAART on levels of Tim-3 expression in Chronic HIV-1 infection. Seven chronically HIV-1-infected individuals from the CIRC cohort were sampled at baseline and at 1, 2, 3 and 6 months post-initiation of HAART. Shown are (a) compiled Tim-3 expression on CD8\(^+\) T cells versus month post-initiation of HAART (b) Tim-3 and CD38 expression levels as determined by flow cytometry, along with absolute CD4\(^+\) T cell count and HIV-1 viral load clinical data. The 6 individuals followed for 6 months achieved undetectable viral loads (bDNA <50 copies/ml). The chart in panel (b) summarizes the p values obtained from a mixed-effects longitudinal analysis studying associations between Tim-3 expression on CD8\(^+\) T cells with: HIV-1 viral load, CD8\(^+\) T cell activation as measured by CD38 expression (MFI), and absolute CD4\(^+\) T cell count. The results of this analysis are further outlined in the text, and details are outlined in methods.

### 7.3.4 - Tim-3 expression defines a population of dysfunctional T\(_{hl}/Tc1\) Cells

We sorted Tim-3\(^+\) from Tim-3\(^-\) populations within both CD8\(^+\) and CD4\(^+\) T cell subsets using PBMC from both HIV-1-infected and uninfected individuals, and quantified T-bet (T\(_{hl}\)), GATA-3 (T\(_{h2}\)), and IFN-\(\gamma\) (T\(_{h1}\)) mRNA by qPCR. For both CD8\(^+\) and CD4\(^+\) T cell populations, GATA-3 was expressed at higher levels in the Tim-3\(^-\) fraction than in the Tim-3\(^+\) fraction, while T-bet was more highly expressed in the Tim-3\(^+\) population (Fig. 7.4). Despite the T\(_{hl}/Tc1\) character of Tim-3\(^+\) cells, we detected the majority of IFN-\(\gamma\) mRNA in the Tim-3\(^-\) CD8\(^+\) population. We then examined IFN-\(\gamma\) and TNF-\(\alpha\) production in response to stimulation with pooled HIV-1-Gag peptides, CMV/EBV/Influenza (CEF) peptides, or staphylococcus enterotoxin B (SEB) in PBMC from 10 acutely/early HIV-1-infected individuals, 10 chronic progressors, 10 viral controllers, and 5 HIV-1-uninfected individuals. In both HIV-1-infected and uninfected subjects, IFN-\(\gamma\) production from CD4\(^+\) and CD8\(^+\) T cells in response to stimulation was observed predominately from the Tim-3\(^-\) population with minimal cytokine production observed in either the Tim-3\(^{lo}\) or Tim-3\(^{hi}\) populations (Fig. 7.5a,b). As a corollary, we identified HIV-1-specific CD8\(^+\) T cells by staining with MHC-I tetramers and observed that in response to cognate peptide IFN-\(\gamma\) was produced only by the Tim-3\(^{lo}\) fraction,
with no IFN-γ production from tetramer⁺Tim-3⁩⁶ cells (Fig. 7.5c). Thus, the lack of cytokine secretion from the Tim-3⁩⁶ population cannot be attributed to an absence of antigen specific cells. Tim-3⁩⁶ CD8⁺ T cells were subsequently sorted from Tim-3⁻⁷⁻ CD8⁺ T cells using ex vivo PBMC from untreated chronic progressors. Both subsets were stimulated with anti-CD3/anti-CD28, and proliferation was assessed by CFSE dilution. Proliferation of the Tim-3⁻⁷⁻ cells was observed, while minimal proliferation was detected in the Tim-3⁩⁶ population (Fig. 7.5d).
**Fig. 7.4.** Quantitative PCR Analysis of T-bet and GATA-3 mRNA in Tim-3⁺ versus Tim-3⁻ T cell subsets. PBMC were stained with monoclonal antibodies to CD3, CD4, CD8, and Tim-3 and sorted into Tim-3⁻CD8⁺, Tim-3⁻CD8⁻, Tim-3⁺CD4⁺ and Tim-3⁺CD4⁻ subsets by flow cytometry. RNA was isolated and reverse transcribed. GATA-3, T-bet, TBP, and IFN-γ transcripts were quantified in triplicate by SYBR real-time PCR. Levels of GATA-3, T-bet, and IFN-γ expression were normalized to TBP. Shown are normalized quantifications expressed relative to the mean of Tim-3⁻CD8⁺ from a representative HIV-1-infected chronic progressor (CIRC cohort). Error bars represent standard error.

**7.3.5 - Tim-3 expression defines a population of dysfunctional Th1/Tc1 Cells**

We sorted Tim-3⁺ from Tim-3⁻ populations within both CD8⁺ and CD4⁺ T cell subsets using PBMC from both HIV-1-infected and uninfected individuals, and quantified T-bet (Tₜ₁), GATA-3 (Tₜ₂), and IFN-γ (Tₜ₁) mRNA by qPCR. For both CD8⁺ and CD4⁺ T cell populations, GATA-3 was expressed at higher levels in the Tim-3⁻ fraction than in the Tim-3⁺ fraction, while T-bet was more highly expressed in the Tim-3⁺ population (Fig. 7.4). Despite the Tₜ₁/Tₖ₁ character of Tim-3⁻ cells, we detected the majority of IFN-γ mRNA in the Tim-3⁻ CD8⁺ population. We then examined IFN-γ and TNF-α production in response to stimulation with pooled HIV-1-Gag peptides, CMV/EBV/Influenza (CEF) peptides, or staphylococcus enterotoxin B (SEB) in PBMC from 10 acutely/early HIV-1-infected individuals, 10 chronic progressors, 10 viral
controllers, and 5 HIV-1-uninfected individuals. In both HIV-1-infected and uninfected subjects, IFN-γ production from CD4⁺ and CD8⁺ T cells in response to stimulation was observed predominately from the Tim-3⁻ population with minimal cytokine production observed in either the Tim-3⁻lo or Tim-3⁻hi populations (Fig. 7.5a,b). As a corollary, we identified HIV-1-specific CD8⁺ T cells by staining with MHC-I tetramers and observed that in response to cognate peptide IFN-γ was produced only by the Tim-3⁻lo fraction, with no IFN-γ production from tetramer⁺Tim-3⁻hi cells (Fig. 7.5c). Thus, the lack of cytokine secretion from the Tim-3⁻hi population cannot be attributed to an absence of antigen specific cells. Tim-3⁻hi CD8⁺ T cells were subsequently sorted from Tim-3⁻lo CD8⁺ T cells using ex vivo PBMC from untreated chronic progressors. Both subsets were stimulated with anti-CD3/anti-CD28, and proliferation was assessed by CFSE dilution. Proliferation of the Tim-3⁻lo cells was observed, while minimal proliferation was detected in the Tim-3⁻hi population (Fig. 7.5d).
Fig. 7.5

(a) (CD8) HIV-1 infected

(b) (CD4) HIV-1 infected

(c) Tim-3

(d) SEB

(e) HIV-1 Uninfected

(f) HIV-1 infected

(g) p < 0.0159

(Pre- and Post-Sorted)
Fig. 7.5. Tim-3 expressing CD8+ and CD4+ T cells populations are dysfunctional. (a,b) PBMCs derived from HIV-1 infected and uninfected individuals were stimulated with pooled peptides or SEB superantigen for 6 hours, and then stained for IFN-γ, TNF-α, and Tim-3 using a polyclonal Tim-3 antibody, and analyzed by multi-parametric flow cytometry. (a) Representative flow cytometry plots showing cytokine responses to pooled Gag peptides and SEB in CD8+ and CD4+ T cells from a chronically HIV-1 infected individual (CIRC cohort). We employed a 3 tiered gating system for analyzing cytokine secretion by Tim-3 expressing cells, considering Tim-3- , Tim-3lo, and Tim-3hi populations. The division between Tim-3- and Tim-3lo populations was determined based on a control normal goat serum staining (as in Fig. 7.1a). The division between Tim-3lo and Tim-3hi was set arbitrarily and then consistently applied to all samples (all run in parallel). (b) Compiled flow cytometry data from 10 chronically HIV-1 infected progressors (CIRC cohort). IFN-γ response percentage for each subset is normalized to the total number of cells within that population. (c) PBMCs from 3 chronic progressors were stained with an HLA-A*0201- SLOTYNTVATL pentamer and stimulated with SLYNTVATL peptide, or DMSO control. Shown are cytokine production and Tim-3 expression as determined by flow cytometry in a representative subject. (d) CD8+ T cells were sorted into purified Tim-3hi CD8+ T cells and Tim-3lo CD8+ T cells populations and labeled with CFSE from one of three representative HIV-1 infected individuals. These two populations were then cultured in the presence of anti-CD3 and anti-CD28 monoclonal antibodies for 5 days. Cells where then assessed for the diminution of CFSE as a read-out of cell division. (e) PBMCs from HIV-1-uninfected (n=5) and HIV-1-infected (n=5) subjects were assessed for levels of intracellular Ki67 antigen expression. Shown are flow cytometry plots displaying Ki67 expression (x-axis) by Tim-3 expression as determined with monoclonal anti-Tim-3 (y-axis). (f) Shown is a histogram presenting Ki67 staining in comparison to isotype controls in Tim-3- and Tim-3+ populations of CD8+ T cells. (g) Shown is compiled Ki67 staining data from 5 HIV-1-infected subjects broken down into Tim-3+ and Tim-3- populations of CD8+ T cells.

7.3.6 - Blocking the Tim-3/Tim-3L pathway enhances the functionality of HIV-1-specific T cells

To delineate the causal relationship between Tim-3 expression and T cell dysfunction, we tested whether blocking the interaction of Tim-3 with its ligand(s) would restore function in Tim-3 expressing cells. We employed a recombinant soluble Tim-3 (sTim-3) glycoprotein to compete for Tim-3 ligands. Addition of sTim-3 enhanced the expansion of CD8+ T cells specific for the HLA-A*0201 restricted HIV-1-Gag epitope ‘SLYNTVATL’ (SL9) in HIV-1-infected chronic progressors in a dose-dependent manner up to 2 µg/ml (Fig. 7.6a). Enhanced proliferation of both CD8+ and CD4+ T cells was also observed when PBMC from chronic progressors were stimulated with pooled Gag and Nef peptides (Fig. 7.6b,c). We corroborated these data by employing a blocking anti-Tim-3 mAb clone (2E2) to disrupt the Tim-3 pathway in an analogous proliferation
assay experiment. Addition of 10µg/ml of mAb 2E2 resulted in a profound rescue of HIV-1-Gag T cell proliferative responses (Fig. 7.6d).

An additional observation from these experiments is that cells which had undergone proliferation in vitro exhibited high levels of Tim-3 expression (data not shown). Tim-3 upregulation in response to anti-CD3/anti-CD28 was observed as early as 20 hours after stimulation, and progressively increased out to at least 120 hours (data not shown). This is consistent with Tim-3 acting as a negative immune regulator, where antigen stimulated cells perform effector functions and then upregulate Tim-3 as a means of terminating responses. In reconciling our ex vivo data showing a lack of cytokine production from Tim-3+ cells with published in vitro data demonstrating an association between IFN-γ production and high levels of Tim-3 expression there is an important distinction to make. Cells expressing Tim-3 ex vivo have been subjected to chronic stimulation in vivo and are dysfunctional to further in vitro stimulation. In contrast, when Tim-3+ cultured cells are stimulated in vitro they perform effector functions, such as produce IFN-γ, and then upregulate Tim-3 to dampen these responses. Thus, depending on when one observes these cultures, high levels of Tim-3 and IFN-γ could be observed in association. This model predicts that in addition to restoring functions of exhausted HIV-1-specific T cells, in vitro treatment with sTim-3 should prolong effector function in response to other antigens. This is supported by examining the level of IFN-γ production at day 5 of in vitro stimulation with anti-CD3/CD28. Under these conditions, all cells that have undergone division express high levels of Tim-3 (data not shown). In the presence
of sTim-3 these cells consistently express higher levels of IFN-γ than in the presence of a control (data not shown).
Fig. 7.6

**a**

![Graph showing the relationship between sTim-3 (µg/ml) and % A2R2.60 T cells](image)

**b**

<table>
<thead>
<tr>
<th>Pool</th>
<th>CD8+ T cells</th>
<th>CD4+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0.49%</td>
<td>0.77%</td>
</tr>
<tr>
<td>Gag+Nef Pool</td>
<td>12.4%</td>
<td>10.7%</td>
</tr>
<tr>
<td>CEF Pool</td>
<td>34.1%</td>
<td>18.8%</td>
</tr>
<tr>
<td><strong>sTim-3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1.52%</td>
<td>2.55%</td>
</tr>
<tr>
<td>Gag+Nef Pool</td>
<td>10.1%</td>
<td>7.5%</td>
</tr>
<tr>
<td>CEF Pool</td>
<td>23.0%</td>
<td>10.6%</td>
</tr>
</tbody>
</table>

**c**

- **CD8+ T cells**
  - **Control**: 0.14%, 0.24%, 0.34%
  - **sTim-3**: 2.74%, 2.11%
  - *p = 0.016*

- **CD4+ T cells**
  - **Control**: 1.7%, 1.71%
  - **sTim-3**: 9.2%, 9.4%
  - *p = 0.047*

**d**

- **CD8+ T cells**
  - **Control**, **anti-Tim-3 mAb (2E2 Clone)**
  - DMSO: 1.78%, 2.67%
  - Gag peptide pool: 4.93%, 92.5%
  - anti-CD3, anti-CD28 mAb: 32.2%, 92.4%
  - CFSE: 0% - 100%

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Fig. 7.6. Addition of soluble Tim-3 enhances the proliferation of HIV-1-specific T cells. (a) PBMC from a chronically HLA-A*0201+ HIV-1 infected individual were stimulated with the SLYNTVATL peptide in the presence of 4, 2, or 1 µg/ml sTim-3, or a control (see Tim-3 expression methods) for 6 days. Cells were stained with HLA-A*0201 SLYNTVATL tetramer, and monoclonal antibodies to CD3, CD8, and anti-Tim-3. Percentages of tetramer+ CD8+ T cells were determined by flow cytometry. Each condition was tested in independent triplicate. Shown are mean percentages of tetramer+ CD8+ T cells on day 6 of stimulation. Error bars represent standard error. (b,c) PBMCs from HIV-1 infected patients were stained with CFSE and the effect of 2 µg/ml sTim-3 on cytokine production and proliferation of PBMCs in response to antigen was determined in these individuals over a 5 day stimulation assay. (b) Shown are representative data from one chronically HIV-1 infected individual on day 5 of culture, showing CFSE (x-axis) by IFN-γ production (y-axis) in CD8+ and CD4+ T cell populations in response to DMSO (upper row), pooled HIV-1 derived Gag/Nef peptides (middle row) or Cytomegalovirus, Epstein-Barr Virus, and Élu Virus (CEF) pooled peptides (lower row) in the presence or absence of either 2 µg/ml sTim-3 or an equal volume of expression control (see Methods). CFSE becomes diluted in cells undergoing proliferation, thus cells in the two left quadrants of each plot have proliferated. (c) Shown are summary data for the effect of 2µg/ml sTim-3 on proliferation in response to Gag from 7 chronically HIV-1-infected individuals. P values were determined by the Wilcoxon matched pairs test. (d) PBMC from an individual with chronic progressive HIV-1 infection were stained with CFSE and stimulated for 7 days with DMSO, pooled HIV-1-Gag peptides, or with anti-CD3 and anti-CD28. For each stimulation the effect of 10µg/ml anti-Tim-3 mAb 2E2 were compared with 10µg/ml murine IgG1 isotype control. Shown are flow cytometry plots showing CD3 by CFSE, where diminution of CFSE is indicative of proliferated cells.

7.3.7 - The Tim-3 expressing T cell population is distinct from the PD-1 expressing population.

Since PD-1 has been identified as a marker of exhausted T cells in HIV-1 infection, we determined whether Tim-3 expression defines the same, or a distinct population. PBMC from 10 individuals with chronic progressive HIV-1 infection were co-stained for Tim-3 and PD-1. Expression was analyzed by flow cytometry after gating on CD8+ or CD4+ T cells (Fig. 7.7). In 9/10 subjects, Tim-3 and PD-1 were primarily expressed by distinct populations of CD8+ T cells. One subject, OM513, displayed a frequent Tim-3+PD-1+ population (23.6%), but retained both Tim-3+PD-1− and Tim-3−PD-1+ populations (23.0% and 16.7% respectively). Similarly 9/10 subjects showed primarily divergent staining for PD-1 and Tim-3 on CD4+ T cells, These data, summarized in Fig.
7.7c,d, clearly demonstrate that Tim-3 expression is independent from PD-1 as a marker of dysfunctional T cells in HIV-1 infection, although co-expression can occur.
Fig. 7.7

(a) Shown are flow cytometry plots from 10 chronically HIV-infected individuals (CIRC cohort), gated on either (a) the CD3+CD8+ population, or (b) the CD3+CD8- population. (c,d) Shown is summary data of the flow cytometry plots displayed in (a,b).
We performed dual staining for Tim-3 and CD25 on both CD4\(^+\) and CD8\(^+\) T cells (Fig. 7.8a). We observed that Tim-3 and CD25 were primarily expressed by distinct populations of T cells. These data demonstrate that Tim-3 expression on CD4\(^+\) T cells does not mark a population of regulatory T cells. We then determined if the Tim-3\(^{hi}\) population could be defined by other cell surface markers that have been used to define the maturation/differentiation status of T cells, by co-staining for CD57, CD45RA, CD27, CD28, and CCR7 (13, 50, 79, 96). Tim-3 expressing CD8\(^+\) T cells from chronically HIV-1-infected individuals were distributed across a range of phenotypic profiles (Fig. 7.8b,c).
Fig. 7.8

**Fig. 7.8. Tim-3 expressing CD8⁺ T cells are present in diverse phenotypic profiles.** (a) PBMCs from HIV-1 uninfected \( (n=3) \) and a chronically HIV-1 infected individuals \( (n=3) \) were labeled with fluorochrome conjugated monoclonal antibodies to CD3, CD8, CD4, CD25, and Tim-3. Shown are flow cytometry plots gated on the CD3⁺CD4⁺ population or the CD3⁺CD8⁺ population from 2 representative individuals as shown. (b) PBMCs were labeled with fluorochrome conjugated monoclonal antibodies against Tim-3, CD3, CD8, CD28, CD27, CD45RA, CCR7 and CD57, as well as with a dead cell discriminating marker. Gating was first performed to include only the viable, CD3⁺CD8⁺ population in subsequent analyses. Shown are phenotypic representations of the Tim-3⁺ populations (blue) versus Tim-3⁻ population (red). (c) Summary data showing phenotypic profiling for 7 chronically HIV-1-infected individuals. Gating for maturation/differentiation markers was determined based on fluorescence minus one controls, and results were analyzed using SPICE software. Shown are the frequencies of populations with the corresponding combination of phenotypic markers, with each individual represented by a single bar.
7.3.8 - Tim-3⁺ T cells exhibit impaired Stat5, Erk1/2, and p38 signaling

We assessed the kinetics of STAT5, Erk1/2 and p38 phosphorylation (pSTAT5, pErk1/2 and p38 respectively) after stimulation in Tim-3^{hi} versus Tim-3^{lo} CD8⁺ T cells in three HIV-1 infected individuals (262). Tim-3^{hi} CD8⁺ T cells had higher levels of basal phosphorylation of STAT5, p38 and ERK1/2 compared to Tim-3^{lo} CD8⁺ T cells, and exhibited lower fold changes in the phosphorylation of these molecules when stimulated in vitro, with: IL-2 for the STAT5 pathway, and PMA/Ionomycin (P+I) for p38 and ERK1/2 (MAP kinase pathway) (Fig. 7.9a,b). This impaired signaling response was seen in every stage of differentiation of Tim-3 expressing cells (Fig. 7.9c-e). Thus, Tim-3 expressing CD8⁺ T cells exhibit a blunted change in phosphorylation of ‘pre-activated’ signaling proteins. This is consistent with the model recently proposed by Schweneker et al in which HIV-1 infection induces chronic activation of T cells resulting in enhanced basal phosphorylation and perturbed signaling in response to restimulation (262). The intracellular domain of Tim-3 contains 5 conserved tyrosine residues, but does not contain sequences corresponding to the ITIM consensus, and its downstream signaling targets remain unknown.
Fig. 7.9

a

CD8

b

Time post-stimulation (min)

Field change phosphorylation

p-Stat5, 45min IL-2

p-Erk1/2, 15min P+I

p-p38, 15min P+I
Fig. 7.9. Tim-3 expressing cells exhibit impaired Stat-5, p35 and Erk1/2 signaling in response to stimuli. Phosphorylation status of Stat5, p38, and Erk1/2 were analyzed by flow cytometry in Tim-3<sup>hi</sup> versus Tim-3<sup>lo</sup> CD8<sup>+</sup> T cells from 3 HIV-1 infected subjects. Whole PBMCs were surface stained on ice, stimulated with either rIL-2 (45min) or PMA/Ionomycin (15min), and phosphorylation of Stat5 or Erk1/2 and p38, respectively, were analyzed with phospho-specific antibodies in CD3<sup>+</sup>CD8<sup>+</sup> T cells and based on their Tim-3 expression. (a) Shown is a representative flow cytometry gating of Tim-3<sup>hi</sup> and Tim-3<sup>lo</sup> CD8<sup>+</sup> PBMCs evaluating the fold change in p38 phosphorylation following 15min of stimulation with a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (P+I) and summary of data from 3 chronically HIV-1 infected individuals, each analyzed in triplicates, with fold changes in phosphorylation of stimulated/unstimulated cells. (b) Shown is a representative time course depicting fold change in phosphorylation (stimulated/unstimulated cells) after 15, 30, and 45min of stimulation. (c-e) Compiled data for (C) Stat5 (D) Erk-1/2, and (E) p38, showing differential levels of change in target phosphorylation (measure by change in mean fluorescence intensity) in Tim-3<sup>-</sup> versus Tim-3<sup>+</sup> cells within each of the following CD3<sup>+</sup>CD8<sup>+</sup> T cell subpopulations: naïve (CD27<sup>+</sup>CD45RA<sup>-</sup>), memory (CD27<sup>+</sup>CD45RA<sup>+</sup>), effector memory (CD27<sup>-</sup>CD45RA<sup>+</sup>), or effector (CD27<sup>-</sup>CD45RA<sup>-</sup>). Statistical analyses were performed using a non-parametric two-tailed Mann Whitney U test. (* P<0.05, **P<0.01,***/P<0.001) were performed using Prism Graphpad.

7.4 – Discussion:

Together, these data support that Tim-3 acts to suppress effector functions of activated T cells in chronic, uncontrolled viral infection with HIV-1. This complements and integrates previous studies which have identified an important role for Tim-3 in immunoregulation, and have implicated defective Tim-3 signaling in the pathogenesis of multiple sclerosis and other autoimmune diseases (10, 149, 302). We show that in HIV-1 infection, the proportion of CD8<sup>-</sup> and CD4<sup>+</sup> T cells in peripheral blood that express Tim-3 can reach in excess of 70% and 30% respectively (in contrast to means of 28.5% and 17.6% in HIV-1-uninfected individuals). As these frequencies exceed the proportion of HIV-1-specific cells in the periphery, suppression of T cell function by Tim-3 likely contributes not only to the loss of functional virus-specific responses, but also to the impairment of responses to other antigens. This is supported by our observations that a subset of CMV and EBV-specific CD8<sup>+</sup> T cells in chronic HIV-1-infected individuals express high levels of Tim-3, and is consistent with observations that HIV-1-infected
individuals have reduced responses to recall antigens and vaccinations (162). The factors leading to this generalized expression of Tim-3 are unknown. Our data does however show a disproportionately high level of Tim-3 expression on HIV-1-specific CD8$^+$ T cells, consistent with the preferential dysfunction of HIV-1-specific CD8$^+$ T cells in chronic HIV-1 infection. We speculate that the heterogeneity which we observed in levels of Tim-3 expression on HIV-1-specific CD8$^+$ T cell responses may reflect the relative functionality of that response, with greater frequencies of Tim-3 expressing antigen-specific cells associated with more advanced exhaustion and dysfunction. It will also be important to determine whether the fixation of escape mutations results in diminished Tim-3 expression on epitope-specific T cells and improvement in functionality, as has been described for PD-1 (274). These questions will be addressed by future studies.

We observed that the initiation of HAART in chronic progressive HIV-1 infection frequently resulted in a decline in Tim-3 expression (4/7 individuals). However, we observed that a subset of chronically HIV-1-infected individuals on HAART therapy (3/7) retained high levels Tim-3 expression despite suppression of HIV-1 viral load to undetectable levels. Maintenance of Tim-3 expression in the context of HAART was associated with sustained high levels of T cell activation (CD38 expression). Persistence of CD38 expression on T cells during HAART are predictive of disease (103, 126, 127, 175). In cases where HAART has failed to result in a reduction in CD38 expression it has been demonstrated that intensification of HAART by eradicating persistent low level
replication can have a positive impact on immunological parameters, including diminishment of CD38 expression (151).

Our demonstration that blockade of the Tim-3 pathway can enhance HIV-1-specific T cell responses \textit{ex vivo} clearly demonstrates that the Tim-3 pathway plays a critical role in suppressing the overall T cell response to HIV-1. It should be noted, however, that although T cell exhaustion is associated with increased viral replication, it is unclear whether this phenotype leads to loss of viral control \textit{in vivo}, or whether this loss results primarily from other factors, such as viral escape from CTL epitopes, or the persistence of viral replication in sanctuaries inaccessible to CTL. The identification of Tim-3 as a novel mechanism of T cell exhaustion constitutes an important pre-requisite for designing studies aimed at delineating the relative contributions of T cell exhaustion versus other factors in the overall inability of the cellular immune response to maintain control of HIV-1 replication. An important implication of the present finding is the possibility that pharmacological agents that block Tim-3 signaling may be of benefit in HIV-1 infection and potentially in other chronic viral diseases. However, it is unclear whether the high level of Tim-3 expressed in HIV-1 infection is the result of a pathological mechanism on the part of the virus to incapacitate the host immune system, or if it is a physiological response to chronic immune activation necessary to hold immunopathology in check. In regards to the latter, recent data supporting that a dysregulation of the Tim-3 pathway may contribute to the pathology of multiple sclerosis highlights the importance of Tim-3 in regulating potentially harmful immune responses (11, 149). This situation is analogous to the considerations required in pursuing PD-1 as a
therapeutic target. Given that Tim-3 expression marks a distinct subset of exhausted T cells from that of the recently identified PD-1 expressing population, it will be intriguing to explore the possibility of an additive effect of targeting both pathways. An important distinction of Tim-3 as a therapeutic target is its unique association with T cells that are impaired not only in their survival and proliferative potential, but also in their ability to produce cytokine. Thus, blockade of the Tim-3 pathway carries the novel potential to enhance not only the numbers of T cells in HIV-1 infection, but also to improve the functionality of both CD8⁺ and CD4⁺ T cells in HIV-1-infected individuals. Since a subset of subjects maintain high levels of Tim-3 expression despite seemingly effective HAART regimens, Tim-3 therapeutics may also play a role in reversing immune defects which persist with HAART.

Acknowledgments. We thank Vijay Kuchroo for generously providing us with the monoclonal anti-Tim-3 antibody ‘2E2’. This research was supported by funds from the Canadian Institutes for Health Research (CIHR), the National Institutes of Health, University of California, San Francisco-Gladstone Institute of Virology & Immunology Center for AIDS Research (P30 AI027763), the UCSF AIDS Biology Program of the AIDS Research Institute (ARI), NIH grants (AI60379, AI68498, AI64520, and AI066917). L.C.N. was supported by the Irvington Institute/Dana Foundation Fellowship from the Cancer Research Institute. M.O. received salary support from the Ontario HIV Treatment Network (OHTN) and the CIHR. R.B.J receives a studentship from the OHTN. M.S. was supported by a grant from the Universitywide AIDS Research Program (F05-GI-219). J.M.M. was supported in part by NIH grant U01 AI43641, by the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research, and by the NIH Director’s Pioneer Award Program, part of the NIH Roadmap for Medical Research, through grant number DPI OD00329. R.K. receives salary support from the Canada Research Chair Program, and grant support from a CIHR Operating Grant #HOP-81735 and an OHTN Operating Grant. Biosafety level 3 laboratory space and equipment was provided by the Canadian Foundation for HIV Research (CANFAR) in partnership with the Canadian Foundation for Innovation and the Ontario Innovation Trust. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (GAG and NEF consensus peptides, CEF pooled peptides) from (DAIDS/NIAID). We would like to thank Mario Roederer (VRC,
NIH) for use of the Spice software for data presentation. RBJ, LCN, DFN, MAO, JR are named as inventors on a patent application, based on this work, which was filed by their respective institutions.

**Author Contributions.** RBJ conceived of the project. RBJ, LCN, JDB, PMS, ARJ, BRL, JCW, MS, MS, JMC, GG, BV, MDH, FYY, AS, DP, JMR designed and performed experiments. ML, RH, DP, GS, FMH, TWC provided clinical samples. JM, RK, JMR, DFN, MAO supervised the project. RBJ wrote the manuscript with input from all co-authors.
Chapter 8: Discussion

The genetic diversity and mutability of HIV-1 represent substantial barriers to the development of vaccines and immunotherapeutics. While a number of strategies with the potential to increase the propensity of vaccine-elicited T cell responses to recognize infecting viruses are currently being developed, it is my opinion that none of these strategies present a viable way to prevent eventual escape upon diversification within an infected individual. This has prompted me to explore a radical new approach to HIV-1 vaccination which targets the immune response against HERV and LINE-1 antigens as surrogate markers of HIV-1-infected cells. This approach represented a high-risk venture for me, as at the time that I decided to pursue it no evidence existed in the literature for the induction of HERV or LINE-1 protein expression in HIV-1-infected cells. I was guided only by the broad idea that the relatedness of exogenous and endogenous retroviruses/retrotransposable elements, including commonalities in their modes of replication and a shared requirement to evade host restriction factors, may present opportunities for interaction. This concept drew some support from a murine precedent, where mobilization of endogenous retroviruses by exogenous ectopic MuLV has been shown to result in the replication of defective endogenous retroviruses (83).

During the first years of my work in this area, there emerged a concrete example of a unifying factor through which HIV-1 could be expected to interact with both HERV and LINE-1 elements. A series of studies presented evidence that members of the APOBEC3 family of proteins, well known in the HIV-1 field for their abilities to restrict replication of Vif-deficient HIV-1, played critical roles in the suppression of LINE-1...
retrotransposition (51, 56, 146, 188, 189, 210, 272, 273). Later, it was demonstrated that some of these proteins – APOBEC3F in particular – were capable of suppressing the replication of reconstituted HERV-K(HML-2) (167). The well described ability for the HIV-1 Vif protein to induce degradation of several members of the APOBEC3 family, including 3F and 3G, comprises a means by which HIV-1 infection could result in the deprepression of both HERV-K(HML-2) and LINE-1 elements. While the discovery of such a mechanism is rewarding, and provides a means for me to focus my initial studies, I resist the temptation to collapse my broad theory onto a single molecular pathway. Instead, I feel that the APOBEC3/Vif interaction is likely to represent just one example of what is likely a complex network of interaction between HIV-1, HERV-K(HML-2) and LINE-1. This is supported by our data which show that, although Vif is necessary for the induction of HERV-K(HML-2) it is insufficient. The systematic delineating of modes of interaction between these elements will reveal ancient conserved mechanisms by which our cells deal with retroviral threats.

In this thesis I explore the effect of HIV-1 infection on LINE-1 and HERV-K(HML-2) expression and activity on a number of fronts, largely dictated by the availability of reagents for these agents. I present qPCR data which clearly demonstrates the induction of both spliced and unspliced HERV-K(HML-2) transcripts upon \textit{in vitro} HIV-1 infection. LINE-1, in contrast, was not induced at the RNA level but, remarkably, displayed striking elevations in the levels of cell associated DNA. Retrotransposition represents an obvious possible explanation for this observation – and indeed we observed elevated levels of retrotransposition in HIV-1-infected cells using an eGFP based reporter
 assay, and sequenced two cases of novel LINE-1 insertion sites in HIV-1-infected cells. However, I cannot conclude, based on this, that retrotransposition and the generation of novel genomic LINE-1 insertions is solely responsible for the massive increases in LINE-1 copy numbers observed over short time frames. Further study, including more advanced techniques for isolating novel LINE-1 genomic insertion sites, is required. As at least some component of this DNA copy number increase can be attributed to retrotransposition, and since LINE-1 retrotransposition is critically dependent upon expression of both LINE-1 ORF1p and ORF2p, I do consider the data presented in Chapter 3 to comprise strong indirect data for the induction of LINE-1 protein expression in HIV-1-infected cells. On the HERV-K(HML-2) side of the story, evidence for HERV-K(HML-2)-Env protein expression in HIV-1-infected cells is provided by immunohistochemistry. Although these experiments were performed to the highest standards, and efforts were made to strictly rule out cross-reactivity of the HERV-K(HML-2)-Env specific antibody with HIV-1 proteins, I do not consider these results to be fully satisfying evidence for the induction of HERV-K(HML-2)-Env expression in the absence of corroborating western blot data. I have therefore made the production of novel HERV-K(HML-2)-specific antibodies a high priority and will employ these reagents in additional western blot and immunoprecipitation experiments to either further support, or to contradict our immunohistochemistry data. The data supporting HERV-K(HML-2)-Gag expression in HIV-1-infected cells, with western blot experiments indicating unique expression of a protein of the correct molecular weight in HIV-1-infected cells. Furthermore I have been able to enhance our sensitivity for detecting HERV-K(HML-2)-
Gag by immunoprecipitating with an anti-HERV-K(HML-2)-Gag pAb and then probing the immunoprecipitate in western blot with an anti-HERV-K(HML-2)-Gag mAb from a different source. I have thus far been unable, however, to pull down sufficient quantities of HERV-K(HML2)-Gag from HIV-1-infected cells to allow for identification of the protein by peptide mass fingerprinting. I am continuing in these efforts with cocktails of high avidity anti-HERV-K(HML-2)-Gag pAbs and mAbs which we have recently produced.

Given the immunological tendencies of our group I have opted, in the meantime, to query the immune systems of HIV-1-infected subjects for indirect evidence of HERV-K(HML-2) and LINE-1 protein expression. Working under the assumption that the expression of these antigens in HIV-1-infected subjects would drive the expansion of T cell responses, I screened a series of HIV-1-infected and uninfected subjects with overlapping 15mer peptides spanning HERV-K(HML-2) Env/Gag and LINE-1-ORF2p. I observed very infrequent T cell responses to these antigens, with responses detected only in two HIV-1-infected controllers. We posit that the general lack of detectable T cell responses could result from one of three factors. First, these antigens may not be expressed in HIV-1-infected cells. Second, the immune system may be tolerized to these antigens and regard them as ‘self’. Third, T cell responses to HIV-1 may be immunodominant to HERV-K(HML-2) and LINE-1-specific T cell responses. In regards to the third possibility, consider that when a cell becomes infected with HIV-1 it expresses both high levels of HIV-1 antigens and relatively low levels of HERV-K(HML-2)/LINE-1 antigens. This is supported by our observation of 2-3 copies of
HERV-K(HML-2) transcripts per copy of TBP versus ~2,500 copies of HIV-1 transcript per copy of TBP (unpublished observations). Given that dendritic cells are a limiting factor in the priming of an immune response, HERV-K(HML-2) and LINE-1-specific T cells may not be able to effectively compete with HIV-1-specific T cells. In the case that high avidity HERV-K(HML-2) and LINE-1-specific T cells are deleted by thymic selection, the remaining low avidity T cells would be even further handicapped. Thus, although this line of inquiry did not provide us with solid evidence for the induction of HERV-K(HML-2) and LINE-1 protein expression in HIV-1-infected cells, it is actually encouraging in terms of our proposal to develop prophylactic and therapeutic vaccines against these antigens. The general lack of HERV-K(HML-2) and LINE-1-specific T cell responses in HIV-1-infected subject presents an opportunity for intervention by inducing such T cell responses in a vaccine platform which focuses the immune response against these antigens. The respective roles of tolerance and immunodominance in this lack of T cell responses does, however, remain an important consideration. The intentional breaking of tolerance against a potential vaccine targeted against a ‘self’ antigen may carry considerable risk and a favourable safety profile would have to be demonstrated in a number of animal models before such a vaccine concept could move towards the clinic.

The clearly demonstrated ability of a HERV-K(HML-2)-Env-specific T cell clone, obtained from the PBMC of an HIV-1-infected controller, to respond to and eliminate cells infected with diverse isolates of HIV-1 represents our strongest evidence to date for both the expression of HERV-K(HML-2) antigens in HIV-1-infected cells, and for the utility of targeting these antigens. My more preliminary evidence
demonstrating the ability of LINE-1-ORF2p-specific T cells to respond to HIV-1-infected cells is similarly encouraging. The DC-based expansion platform, after extensive optimization, is starting to bear fruit in the form of HERV-K(HML-2)-specific T cell responses that were present below the limit of detection \textit{ex vivo}. I intend to focus considerable efforts on this platform in order to determine whether the observed recognition and elimination of HIV-1-infected cells by HERV-K(HML-2)-specific T cells is truly generalizable, and whether the presence of low-level T cell responses is associated with control of natural HIV-1-infection. Similar efforts will be made with LINE-1-ORF2p, but presently are lagging behind due to technical issues. Even should my proposed strategy of vaccinating individuals with HERV-K(HML-2) and LINE-1 antigens to prevent or control HIV-1-infection prove inviable (for ex. due to safety concerns) it is vital to determine whether these responses may play a role in the phenomenon of natural control of HIV-1 infection. At present, such natural control remains a mystery. It is clear that such control is T cell mediated, however any attempts to correlate magnitude or breadths of HIV-1-specific T cell responses with immune control have failed. It is most tempting to speculate that the relevant T cell responses may, in fact, not be directed against HIV-1 antigens at all.

It is worth taking a moment to consider why, in 30 years of research, a potential interaction between HIV-1 and endogenous retroviruses/retroelements has been overlooked. There are a couple of factors which may have lead to this. First is the use of the lab-adapted isolate of HIV-1 ‘NL4-3’ in the majority of studies aimed at delineating host interactions. Our data from qPCR, protein expression and T cell clone recognition
converge in indicating that NL4-3 induces lower levels of HERV-K(HML-2) expression as compared to primary isolates of HIV-1. The mechanisms underlying this observation are presently unclear, but I suggest that the adaptation of NL4-3 to leukemic cell lines, where host restriction factors are generally compromised, may have led to reduced capacity of the virus to counter such factors. As an example, it has been shown that, while NL4-3 efficiently induces the degradation of APOBEC3G it has no effect on cellular levels of APOBEC3F. A second factor which may have led the field to miss interactions between HIV-1 and HERV/LINE-1 is that many of the assays used to systematically screen for host factors that are specifically affected by HIV-1-infection exclude consideration of HERV and LINE-1 elements, which have long been grouped under the banner of ‘junk DNA’. Microarrays, for example, omit repetitive element sequences – including HERV and LINE-1. As another example, in BLAST searches HERV sequences are not considered to be ‘human’, thus a search with a HERV DNA or RNA sequence refined to our species will turn up blank. Databases queried for mass spectrometry peptide mass fingerprinting are similarly deficient for HERV sequences. In this thesis we present the possibility that one man’s junk can be another man’s treasure.

While the primary focus of this dissertation has been exploring the potential to utilize HERV-K(HML-2) and LINE-1-specific T cell responses to circumvent the challenge of HIV-1 sequence diversity, I have also embarked upon an exploration of the factors underlying a second limitation of cellular immunity – that of T cell exhaustion. I have provided the first demonstration that the Ig superfamily molecule Tim-3 plays a causal role in HIV-1-induced T cell exhaustion. Remarkably, greater than 70% of total
CD8⁺ T cells express Tim-3 in some chronically HIV-1-infected subjects - speaking to a role for Tim-3 in generalized HIV-1-related immune dysfunction, in addition to HIV-1-specific exhaustion. I demonstrate that blocking the Tim-3 pathway \textit{in vitro}, through treatment with either a soluble Tim-3 or an anti-Tim-3 antibody resulted in enhanced functionality (proliferation, cytokine production) of virus-specific T cells. My work in this area has been extended by others into a number of other viral infection systems including: herpes simplex virus, hepatitis C virus, hepatitis B virus, human T lymphotrophic virus, and Friend virus (4, 105, 140, 199, 283, 298). Furthermore, in a study that agrees with our unpublished data from the same system, blockade of Tim-3 \textit{in vivo} has been shown to work synergistically with blockade of the negative regulator PD-1 to restore T cell function and enhance viral clearance in LCMV-infected mice (133). In thinking about potential clinical applications of these findings, I envision a strategy where Tim-3 is blocked in conjunction with a therapeutic vaccine in chronic HIV-1 infection to boost and/or re-focus the T cell response. One potential manifestation of this, with particular relevance to this dissertation, would be the combination of Tim-3 blockade with a vaccine which elicits HERV-K(HML-2) or LINE-1 specific T cell responses. Alternatively, Tim-3 blockade in combination with a vaccine with elicits T cell responses to specific HIV-1 antigens or epitopes could be used to re-focus immune responses against desirable targets, such as against a conserved region of Gag. The development of such combination approaches carries significant technical and regulatory hurdles. However, such strategies may be required to achieve the ambitious goals of
either eradicating HIV-1 within an infected individual, or establishing vaccine-mediated long-term control of viral replication.

Chapter 9: Future Directions

9.1 – Optimization of new HERV-K(HML-2) and LINE-1 specific antibodies in HIV-1-infected cells.

Although the data presented in this dissertation are strongly supportive of the induction of HERV-K(HML-2) and LINE-1 antigen induction in HIV-1-infected cells, with multiple lines of evidence converging on this point, I feel that stronger direct evidence for the expression of these proteins in HIV-1-infected cells is needed. I have therefore produced high quality recombinant HERV-K(HML-2) and LINE-1 proteins and initiated the production of both polyclonal (rabbit) and monoclonal (murine) antibodies. These reagents will be validated and optimized for western blot, immunoprecipitation and flow cytometry applications using cell lines transfected with the corresponding antigens, and then will be applied to a series of HIV-1-infected and uninfected primary cells. I am particularly interested in using these reagents to immunoprecipitate HERV-K(HML-2) and LINE-1 proteins from HIV-1-infected cells for identification using mass spectrometry techniques. The confirmation of the identities of these proteins would not only provide unambiguous evidence for their presence in infected cells, but would also assist us in identifying precise HERV-K(HML-2) and LINE-1 genomic loci that are induced at the protein level in HIV-1-infected cells.
9.2 – Delineating mechanisms of HERV-K(HML-2) and LINE-1 induction in HIV-1-infected cells

Thus far, I have determined that the expression of HIV-1-Vif is necessary, but not sufficient, for the induction of HERV-K(HML-2) antigen expression, as measured by T cell recognition. Similarly, I have observed that the Vif protein is necessary for the induction of LINE-1 retrotransposition. I am currently working to determine whether Vif is necessary for the observed recognition of HIV-1-infected macrophages by our LINE-1-ORF2p-specific T cell clone, as well as to determine whether Vif is sufficient for the induction of LINE-1 retrotransposition and for this recognition. Given the high degree of success we have had in inducing expression of Vif in primary CD4+ T cells by electroporation of corresponding mRNA, we are working to extend this platform to other HIV-1 gene products including: Vpr, Vpu, Nef, Tat and Rev. After confirming expression of each of these proteins individually, we will proceed to test different combinations in T cell clone recognition assays and in LINE-1 retrotransposition assays in order to determine the minimal requirements for induction of HERV-K(HML-2) and LINE-1 antigen expression. I will then confirm these results by studying these target cells for direct evidence of protein expression using the antibody reagents discussed above. Upon identifying the minimal set of proteins needed to induce HERV-K(HML-2) and/or LINE-1 antigen expression we will proceed to modify key sites of the relevant HIV-1 proteins in order to more precisely define mechanism. For example, mutations will be made to key APOBEC3F and/or 3G interacting domains in HIV-1-Vif in order to determine whether this abolishes HERV-K(HML-2) and/or LINE-1 induction. In addition to increasing our general knowledge regarding this interaction between host – endogenous retrovirus /
retroelement – exogenous virus, this will provide us with a valuable means of precisely replicating the depression of HERV-K(HML-2)/LINE-1. One could envision a vaccination or immunotherapeutic strategy whereby, rather than immunizing directly with HERV-K(HML-2)/LINE-1 antigens, the vaccine vector delivered the required HIV-1 viral factors to indirectly induce expression of the relevant subset of HERV-K(HML-2)/LINE-1 antigens in the context of appropriate adjuvant.

9.3 – Determination of precise HERV-K(HML-2)/LINE-1 loci induced upon HIV-1-infection.

In developing a vaccination strategy that aims to overcome with genetic diversity of HIV-1, it is important to not neglect the genetic diversity of the endogenous elements that I plan to target. It is reasonable to speculate, for example, that the complement of HERV-K(HML-2) loci within the human genome can be divided into three categories based on expression potential: i) insertions that cannot ever be expressed at the protein level – for example due to the lack of a suitable poly(A) signal ii) insertions with ORFs that are expressed at some level in a variety of circumstances – both in health and disease iii) insertions for which expression is preferentially induced in the context of HIV-1-infected cells. Thus if I were to immunize with a general HERV-K(HML-2) consensus sequence, with no knowledge as to which insertions were specifically induced in HIV-1-infected cells, I would expect that this consensus sequence would comprise a chimera of T cell determinants representing each of these three types of insertions. In the case that there is even a small level of tolerance to insertions representing categories ii and iii, the immune response would be expected to focus on determinants which were completely
lacking from these insertions (but made their way into the consensus sequence due to the inclusion of insertions from category i). The result of this would be that HERV-K(HML-2)-specific T cell responses would be generated upon immunization, but that such responses would be completely ineffective in recognizing HIV-1-infected cells. Unlike the genetic diversity of HIV-1, the genetic diversity of HERV-K(HML-2) in the human genome is stable (at least over the time-lines relevant to vaccine development and implementation). The sequences of induced HERV-K(HML-2) antigens are predictable, and not prone to the acquisition of escape mutations. It is, however, critical that an effort is made on the front-end to identify precise antigen targets. I propose two approaches to this objective. First, in addition to studying changes in total levels of HERV-K(HML-2) mRNA sequences induced in HIV-1-infected cells, I will proceed to perform bulk sequencing both pre and post HIV-1 infection. Comparative phylogenetic analysis will allow for the identification of branches which are disproportionately represented in HIV-1-infected cells. The identification of precise genomic loci by this method is likely to result from an iterative process where, upon focusing in on a narrower subset of HERV-K(HML-2), primers will be designed to amplify regions which will allow for finer discrimination of genomic loci within this subset. Secondly, I propose that the identification, by mass spectrometry, of HERV-K(HML-2) proteins, immunoprecipitated from HIV-1-infected cells, will allow for direct identification of antigenic targets expressed at the protein level. This latter approach is clearly more direct, but is contingent upon the ability to immunoprecipitate sufficient proteins from infected cells. It is worth noting that although I have focused on the HML-2 family of HERV-K, I cannot
rule out at this point that our data are actually indicative of induction by a related lineage (such as HML-3). It is very feasible that our primers/probes, antibodies, and T cell clones could be cross-reactive against such related lineages. The proposed phylogenetic and proteomic approaches to identifying antigen targets will consider this possibility. Note also that, while I have focused on the need to identify precise targets of HERV-K, these methods should also be applied to LINE-1. Given the much greater abundance of LINE-1 insertions, it is less likely that I will be able to pin-point exact genomic loci that are induced upon HIV-1-infection. However, the proposed techniques should allow us to identify specific LINE-1 lineages as targets.

**9.4 – Evaluating safety considerations of HERV-K(HML-2) and LINE-1 based vaccination in humans in regards to potential for inducing autoimmunity.**

In proposing to induce immune responses against antigens encoded in the human genome, it is important to consider the possible dangers of eliciting autoimmunity inherent in this approach. Extensive studies by my colleagues at Pfizer Drug Safety R&D using available anti-HERV-K(HML-2) and LINE-1 antibodies have failed to produce any evidence for expression of these proteins in a diverse panel of healthy tissues. It is impossible, however, to prove that this indicates a total lack of expression rather than expression below the limits of sensitivity of these assays. We envision drawing from two primary lines of evidence in regards to evaluating the safety of the proposed vaccine approaches. Firstly, I am gathering extensive clinical histories, and performing autoimmunity panels on subjects, for whom I am screening for HERV-K(HML-2) and LINE-1 specific T cell responses in our DC expansion assays. Once I have completed
screening these subjects I will examine our data for indications of correlations between the presence of these T cell responses and any autoimmune indications. I draw some early encouragement from the observation that subject ‘OM9’ from whom I have obtained our HERV-K(HML-2)-Env-specific T cell clone, and in whom I have identified a HERV-K(HML-2)-Gag-specific T cell responses by DC expansions, does not have any clinical history of autoimmune disorders. Secondly, upon testing my vaccine approach in non-human primates (NHP), these animals will be monitored extensively for signs of autoimmunity both while alive and in necropsy. Although I assert that these methods can be used to substantially de-risk the proposed vaccination strategy, I acknowledge that first-in-man tests may be performed in the context of therapeutic vaccination in HIV-1-infected subjects, and that a strong safety record in this context may be required before applying the vaccination in a prophylactic setting. As a final note, the background work proposed in 9.3 to focus on very specific HERV-K(HML-2) and LINE-1 lineages and genomic loci is critically related to an assessment of safety. At present, we note that HERV-K(HML-2) mRNA, as detected using our broadly targeted primers, is ubiquitously present in uninfected cells and simply induced to higher levels upon HIV-1 infection. Given the sensitivity of PCR, the identification of precise genomic loci which are exclusively detectable at the mRNA level in HIV-1-infected cells, would support the idea that such insertions could be targeted safely.
9.5 – Evaluating endogenous retrovirus (ERV) and LINE-1 induction in SIV/rhesus macaque model.

The pathway to clinical development of a HERV-K(HML-2) and/or LINE-1 targeted HIV-1 vaccine would involve the critical step of a challenge study in the non-human primate (NHP) rhesus macaque / SIV infection model. This may pose a substantial hurdle, as it is unclear whether the induction of HERV-K(HML-2) and LINE-1 would be recapitulated in this system. We note that SIVmac infection of human cells does result in the induction of HERV-K(HML-2) antigen expression as measured by T cell recognition. However, without a better knowledge of the mechanisms by which antigen expression is induced in human cells, we cannot predict whether this will be conserved in the rhesus macaque. Furthermore, given that a subset of HERV-K(HML-2) insertions are human-specific, whether induction is recapitulated in the rhesus macaque may depend on whether the relevant insertion loci are shared. Before moving towards a challenge study, it will be critical to apply the methods proposed in 10.3 to identify precise HERV-K(HML-2) and LINE-1 antigen targets that are induced in SIV-infected rhesus macaque primary CD4+ T cells. Although we propose focusing on the SIV/rhesus macaque model, as it is generally considered the gold-standard for evaluating HIV-1 vaccine concepts, we also note the availability of other NHP models including cynomologous pig-tailed macaques. Should we be unable to detect the induction of HERV-K(HML-2) and LINE-1 expression in SIV-infected rhesus macaque cells then these other models should be considered. It is also conceivable that our vaccine strategy could be tested in humanized mouse models of HIV-1 infection. At present, technical
hurdles preclude the testing of T cell mediated vaccine concepts in humanized mice, but work is underway to facilitate such studies.

9.6 – Testing the effect of blocking Tim-3 in the rhesus macaque SIV infection model.

Given that we, and others, have now established that Tim-3 blockade improves T cell function and viral clearance in the LCMV murine model of chronic viral infection, the next logical step towards clinical development is to test whether Tim-3 blockade is of benefit in the rhesus macaque SIV infection model. Such studies were recently undertaken for the T cell exhaustion marker PD-1, where blockade resulted in the rapid expansion of virus-specific T cells with improved functionality, reductions in plasma viral load, and prolonged survival (286). We are currently in the process of establishing collaborations to pursue this work.
In closing, while I will continue to pursue the development of a HERV-K(HML-2) and/or LINE-1 targeted vaccine against HIV-1, I acknowledge the magnitude of the obstacles facing this approach, and the multitude of ways that this approach could fail. What I am hoping to have accomplished, if nothing else, is to have awakened the field to the ongoing possibility for innovation. There has been a prevailing sense of frustration in the HIV-1 vaccine field, with some senior scientists voicing despair that a vaccine may never be possible. The idea presented in this thesis is intuitive, yet was untouched. I suspect that the HIV-1 pandemic will be eradicated well before the potential for novel approaches to vaccination are exhausted.
References


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