IMMUNOGENICITY OF DRUG RESISTANT HIV

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by

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Abstract

Antiretroviral drug resistance and cytotoxic T lymphocyte (CTL) escape are considered to be major obstacles to effective control of human immunodeficiency virus (HIV). To investigate the possibility of combining drug and immune-based selective pressures to combat the emergence and transmission of HIV, we studied the effect of drug resistance mutations on CTL recognition of HIV-1 *pol* epitopes. Several drug resistance mutations sustained or even enhanced antigenicity and immunogenicity of HIV-1 epitopes. Thus, drug resistant HIV is susceptible to immune selective pressure which could be applied to combat transmission or emergence of drug resistance and enhance the immune response against HIV. We also observed instances of activation of CTL against a control self-peptide derived from interferon-gamma inducible protein (IP)-30 following *in vitro* stimulation with an HIV protease (PR) peptide. We found that activation of IP-30-specific CTL resulted from T cell cross-reactivity between the two peptides and indicates that HIV PR 76-84 peptide is a heteroclitic peptide variant (i.e. more immunogenic variant) of the IP-30 signal peptide, which may have implications for immune memory and autoimmunity.

Co-Authorship Statement

The work described in Chapter 2 has been published as a manuscript entitled *Antiretroviral Drug Resistance Mutations Sustain or Enhance Recognition of Common HIV-1 Pol Epitopes* in *The Journal of Immunology,* 2004, 172: 7212–7219. This project was identified and outlined by Dr. Michael Grant. I was responsible for practical aspects of the research and the experiments were primarily conducted by myself, while the data analysis and manuscript preparation were done jointly with Dr. Michael Grant. Dr. Ian Bowmer and Ms. Constance Howley assisted with clinical aspects including donor testing, recruitment and sample collection. Ms. Jennifer Myers conducted some preliminary cytotoxicity experiments while immortalized donor B cell lines used in this study were generated by Ms. Maureen Gallant.

The work outlined in Chapter 3 has been published as a manuscript entitled *Cross-reactive Cytotoxic T Lymphocytes Against Human Immunodeficiency Virus Type 1 Protease and Interferon-gamma-inducible Protein 30* in *Journal of* Virology, 2005, 79: 5529-5536. This project was identified and designed with the help of Dr. Michael Grant. I completed most of the experiments described, while the data analysis and manuscript preparation were done jointly with Dr. Michael Grant. Dr. Ian Bowmer and Ms. Constance Howley assisted with clinical aspects including donor testing, recruitment and sample collection. Immortalized donor B cell lines used in this study were generated by Ms. Maureen Gallant.

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I take from my MSc experience many good memories, friendships and best of all, my cat Alex.

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List of Abbreviations and Symbols

3TC	lamivudine
aa	amino acid
Ab	antibody
ABC	abacavir
Ag	antigen
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
APC	antigen presenting cell
ART	antiretroviral therapy
AZT	zidovudine
BLCL	B lymphoblastoid cell line
BSA	bovine serum albumin
CCR5	chemokine (C-C) receptor 5
CD	cluster of differentiation
Cr	chromium
CRF	circulating recombinant form
CTL	cytotoxic T lymphocyte
CXCR4	chemokine (C-X-C) receptor 4
d4T	stavudine
DDC	zalcitabine
DDI	didanosine
DNA	deoxyribonucleic acid

EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISPOT	enzyme-linked immunospot assay
env	envelope
E:T	effector to target ratio
FACS	fluorescence-activated cell sorting
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
gag	group specific antigen protein
GILT	γ-IFN-inducible lysosomal thiol reductase
gp	glycoprotein
HAART	highly active antiretroviral therapy
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HXB2	HIV-1 isolate from France
Ig	immunoglobulin
IL	interleukin
IFN-γ	interferon gamma
IP-30	interferon gamma inducible protein 30
LTNP	long-term nonprogressor
LTR	long terminal repeat

mAb	monoclonal antibody
MHC	major histocompatibility comples
MIIC	MHC class II compartment
NNRT	nonnucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrine
PHA	phytohemagglutinin
PI	protease inhibitors
pol	polymerase
PR	protease
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SFC	spot forming cell
SIV	simian immunodeficiency syndrome
vCF21	vaccinia virus expressing entire HIV-1 clade B HXB2 Pol protein
vSC8	vaccinia virus expressing Escherichia coli β-galactosidase

1. Introduction and Overview

1.1. History of HIV / AIDS

In 1981, the first case of acquired immune deficiency syndrome (AIDS) was reported in a healthy homosexual male with no previous history of immune deficiency (1). Shortly thereafter, Barre-Sinoussi and colleagues isolated the human immunodeficiency virus (HIV) type 1, the aetiologic agent of AIDS, from a patient at risk for AIDS (2, 3). Over the past twenty years, considerable resources and research have been expended to address the HIV pandemic. Unfortunately, despite progress and advances in understanding the biology of HIV, as well as its immunopathology and the host immune response against it, a HIV vaccine has yet to be developed. Furthermore, while antiretroviral therapy (ART) has been beneficial in reducing morbidity and mortality, the accumulation of drug resistance mutations in treated individuals remains a significant obstacle to effective long-term control of HIV. Current information on HIV is summarized in the following sections of this introduction.

1.2. Biology of HIV

1.2.1. Virus Classification

HIV is a lentivirus of the retroviridae family of viruses. These viruses possess the enzyme reverse transcripase (RT), which is responsible for reverse transcribing the viral ribonucleic acid (RNA) genome into deoxyribonucleic acid (DNA) for integration into the host cell genome. Its classification as a member of the lentivirus genus reflects the long incubation period between primary HIV infection and development of AIDS.

1.2.2. Virus Structure

The mature HIV virion (Figure 1), approximately 80-100 nm in diameter, is enveloped by a lipid bilayer derived from the host cell membrane. Anchored to this membrane are envelope glycoproteins, i.e., gp120, a surface molecule (SU), and gp41, a transmembrane protein (TM), which are involved in virus attachment and entry (4). Since HIV acquires its membrane by budding from infected cells, several host cell-derived proteins such as major histocompatibility complex (MHC) class I and II antigens are also found on the surface of the mature virion. The nucleocapsid core contains two copies of the plus sense single stranded (ss) RNA genome stabilized by the nucleocapsid protein p7 (NC). The core also encapsulates the viral enzymes reverse transriptase (RT), integrase (IN) and protease (PR).

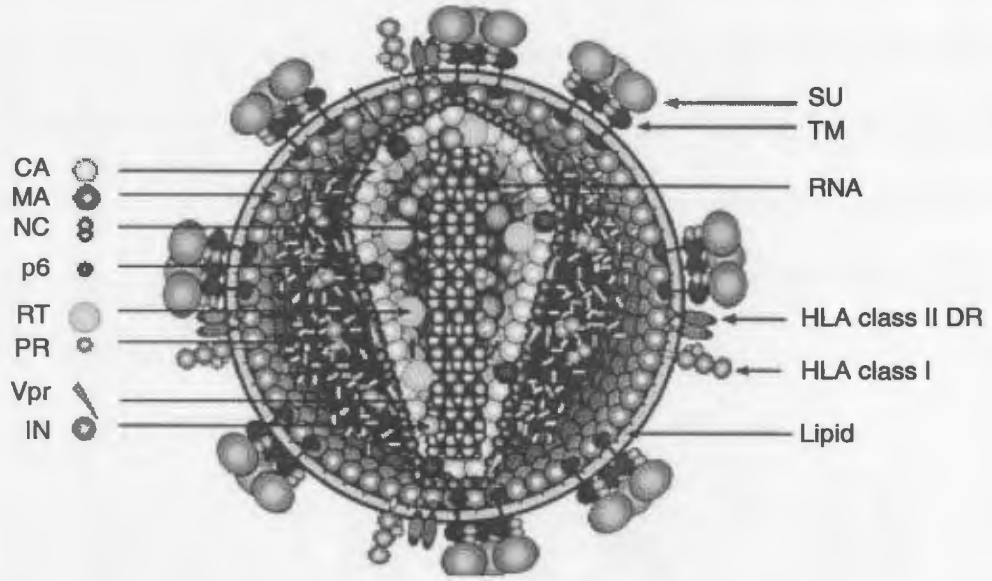

Figure 1. Schematic representation of mature HIV-1 virion [adapted from Coffin, J. M., Cold Spring Harbor Laboratory. Press. 2002. Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY]

1.2.3. Viral Genome

The genomic organization of HIV is depicted in Figure 2. The HIV genome is approximately 10,000 bases. All simple retroviruses contain three common essential genes: gag, pol, and env, which encode core proteins, viral enzymes and structural proteins, respectively. Complex retroviruses, such as HIV, encode additional regulatory proteins. HIV encodes six accessory proteins: i) **tat** promotes processivity of transcription by RNA polymerase complex II (5, 6); ii) **rev** is responsible for nuclear export of unspliced viral RNA transcripts (7); iii) **nef** selectively downregulates MHC class I antigens to prevent cytotoxic T lymphocyte (CTL) and natural killer (NK) cell-mediated lysis of infected cells (8); iv) **vif** inhibits packaging of cellular cytidine deaminase apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) into HIV virions, thereby increasing virus infectivity (9); v) **vpr** enhances nuclear transport of viral nucleic acids in non-dividing cells (10); and vi) **vpu** enhances virion release from infected cells and interacts with and induces CD4 degradation (11-13). Genetic sequences termed long terminal repeats (LTR) flank the ends of the viral genome and play an important role in regulating viral gene expression and guiding integration into host cell DNA.

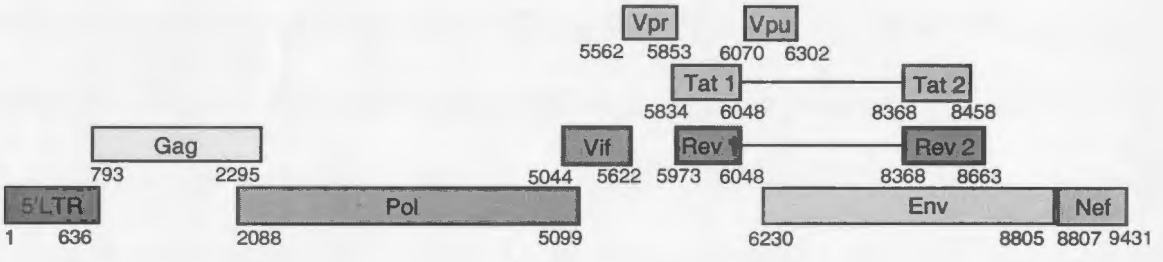

Figure 2. Genomic organization of HIV-1 [adapted from http://www.hiv.lanl.gov/content/hiv-db/MAP/hivmap.html].

1.2.4. HIV Replication

Recognition of the target cell by mature HIV virions occurs via an initial interaction between the envelope glycoprotein gp120 and the CD4 molecule (14-16). This interaction causes a conformational change that exposes multiple sites on gp120 (17, 18) involved in binding to one of several chemokine receptors, including CCR5 or CXCR4 (19-21). Attachment of gp120 to its CD4 receptor and co-receptor induces further conformational changes in gp41, which promote fusion of viral and target cell membranes (22-24).

Following entry and uncoating of the virus, reverse transcription begins within the nucleoprotein complex. The enzyme RT contains both polymerase and ribonuclease (RNase) H domains required for transcribing viral RNA into double stranded (ds) DNA. The polymerase domain consists of four subdomains referred to as "fingers", "palm", "thumb" and "connection" (25) which assume an "open right-hand configuration" to form the polymerase active site. Minus strand DNA synthesis is initiated using host-derived $tRNA_{Lys}$ packaged within mature HIV virions as a primer. RNase H then cleaves viral RNA to remove it from the RNA-DNA hybrid and generate the primer used to initiate plus strand DNA synthesis. Completion of the plus strand DNA yields a complete dsDNA copy of the viral RNA. The nucleoprotein complex containing this dsDNA and the enzyme integrase enters the nucleus and facilitates integration of viral and host cell DNA. Ligation of the ends of viral and host DNA completes the irreversible integration process. Since transcription of proviral DNA does not usually occur in resting cells, integrated proviral DNA can remain latent indefinitely.

In activated cells, transcription of proviral DNA is mediated by host cell RNA polymerase II and results in production of both spliced and unspliced full-length transcripts which are transported to the cytoplasm for protein translation. Full-length transcripts encode gag and pol gene products and serve as genomic RNA for progeny virions while single and multiply spliced transcripts encode the remaining viral proteins (26-31). As HIV viral proteins are synthesized and viral replication proceeds, full-length transcripts begin to accumulate within the cytoplasm for packaging into progeny virus. Protease is responsible for post-translational processing of GagPol polypeptides, which releases proteins required for virion assembly, while Gag directs budding of virus particles from the plasma membrane.

1.3. Antiretroviral Therapy

1.3.1. Protease Inhibitors

Protease-mediated cleavage of GagPol polypeptides is essential for virion assembly and infectivity, thus rendering PR a key target for inhibition by antiretroviral drugs. Protease inhibitors (PI) target the active site of PR, a symmetrical homodimer with structural similarity to aspartyl proteases.

1.3.2. Reverse Transcriptase Inhibitors

Conversion of viral RNA into DNA by RT for subsequent integration into the host cell genome is another essential step in HIV replication that is inhibited by drugs belonging to one of two classes of RT inhibitors: nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). NRTI are chain terminating nucleoside analogues that compete with their natural counterparts for integration into nascent DNA (32). NNRTI inhibit RT by interacting with a pocket in

RT that is distinct from the active site, thus causing a conformational change in the polymerase active site which impairs enzyme function (33, 34).

1.3.3. Fusion Inhibitors

Since the fusion process is an essential part of the viral replication cycle it represents an additional target for inhibition of HIV replication. The only fusion inhibitor currently in clinical use is Enfuvirtide, also known as T-20, although additional synthetic peptides with similar function are being developed. Enfuvirtide is a synthetic peptide of the extracellular domain of gp41 and inhibits the conformational change required for fusion of viral and host cell membranes (35, 36).

1.3.4. Integrase Inhibitors

There are currently two classes of integrase inhibitors in development; the diketo acid inhibitors (DKA), which specifically inhibit the DNA strand transfer step of the HIV integration process and 5*H*-pyrano[2,3-*d*:-6,5-*d*'] dipyrimidines (PDPs), which interfere with formation of the DNA-integration complex (37-39).

1.4. Immune Response to HIV

1.4.1. Primary Infection

During the initial stage of HIV infection, known as primary infection, viral RNA can be detected in the plasma of infected individuals within a few days of exposure to HIV. Clinical symptoms resembling mononucleosis often appear within 2 to 6 weeks (40). Primary infection is usually characterized by a massive burst in viremia and a marked increase in the level of circulating infected cells (41). Control of viremia during primary infection is mediated principally by CTL, since decrease in viremia occurs coincident with the emergence of anti-HIV CTL (42, 43) before neutralizing antibodies

are detectable (44). Despite this vigorous CTL response, HIV is not eliminated, although the amount of virus decreases and eventually stabilizes at a level referred to as the "viral set point". Viral set point is a strong predictor of the rate of clinical disease progression (45).

1.4.2. Clinical Latency

Establishment of the viral set point produces a prolonged asymptomatic period that can last several years. Although the level of HIV RNA remains undetectable in plasma, there is continuous low-level viral replication, as well as progressive loss of $CD4^+$ T cells during this asymptomatic stage. Moreover, since HIV is able to establish latent infection by integrating into genomic DNA, reservoirs of latently infected cells allow HIV to survive indefinitely (46).

1.4.3. Acquired Immune Deficiency Syndrome

The final stage of HIV infection is characterized by a marked increase in virus load with viral replication occurring in both lymphoid and non-lymphoid tissues (47, 48). As the $CD4^+$ T cell count falls below 200 cells /μl, clinical immunodeficiency becomes a greater risk and opportunistic infections are the eventual cause of death in most AIDS patients.

1.4.4. Long-Term Non-Progressors

Most untreated HIV-infected individuals eventually progress to AIDS, yet some individuals, termed long-term non-progressors (LTNP), are able to control virus and maintain high $CD4^+$ T cell counts for prolonged periods in the absence of antiretroviral treatment (49). For some LTNP, attenuated disease course is due to polymorphisms in

HIV that diminish viral pathogenesis (50-53). However, infection with less virulent strains of HIV does not account for most LTNP. Slow disease progression is more often linked with host genetic factors such as polymorphisms in the chemokine receptors used for entry by HIV (54-56) or possession of specific human leukocyte antigen (HLA) alleles (57-59). The strong association between particular HLA class I alleles and slow disease progression suggests that $CD8^+$ T cell recognition of HIV antigens is crucial for immune control of HIV.

1.4.5. Resistance to HIV

Anti-HIV CTL responses not only help control established HIV infection but may also facilitate resistance to infection. HIV-specific CTL have been detected in individuals who apparently resist infection despite repeated exposure to HIV (60-63). Resistance to HIV has also been associated with HIV-specific humoral immunity (64-66) and in some instances with homozygosity for a 32-base pair deletion mutation in the CCR5 allele (Δ32-CCR5) that prevents cell-surface expression of the chemokine receptor used for entry by most primary isolates of HIV (67, 68). However, since the frequency of homozygosity for Δ32-CCR5 is approximately 1% in Caucasian populations and this mutated allele is absent in non-Caucasian populations, (69) Δ32-CCR5 cannot account for the majority of individuals who resist HIV infection. For this reason, there is a strong focus on identifying HIV-specific CTL responses responsible for mediating resistance to HIV infection and resistance to disease progression.

1.5. Viral Escape

1.5.1. CTL Escape Mutations

Since HIV-specific CTL play a crucial role in suppression of viremia, they exert strong selection pressure on the virus. Rapid virus turnover coupled with a highly error-prone HIV RT that introduces approximately one nucleotide substitution per genome per replication cycle (70, 71) generally results in the emergence of CTL escape mutations. Mutations conferring escape from CTL may occur at sites that affect MHC binding (72), T cell receptor (TCR) recognition (73) or antigen processing (74) and can lead to loss of immune control of HIV (72, 75).

1.5.2. Drug Resistance

Uncontrolled viral replication also follows emergence of drug resistance mutations. NRTI were the first drugs used in mono or dual antiretroviral therapy to treat HIV. However, treatment often failed due to the rapid emergence of drug resistance mutations. With the introduction of NNRTI and PI, antiretroviral therapy (ART) was expanded to include combinations of three or more inhibitors. Treatment with a potent combination of anti-HIV compounds, also termed highly active antiretroviral therapy (HAART), was shown to reduce and maintain plasma viremia at undetectable levels for several years (76) and has now become the standard treatment for HIV-infected individuals. Unfortunately, prolonged exposure to HAART selects for drug resistance mutations which lead to viral rebound and progression to AIDS. During HAART, mutations that allow viral replication to continue in the presence of drugs are selected. Although these mutations may sometimes result in reduced viral fitness compared to wild-type virus, subsequent mutations compensating for any reduction in viral fitness are

also eventually selected. This results in high level resistance and or cross-resistance to multiple drugs, thus limiting the effectiveness of HAART.

1.6. Study Objectives

1.6.1. CTL Recognition of Drug Resistant HIV

While CTL responses and antiretroviral drugs are effective at controlling HIV replication, they ultimately fail to prevent disease progression when escape mutations emerge. The idea of more durably limiting viral replication by combining immune and drug selection pressures is supported by the strong selection for escape mutations that both CTL immune responses and antiretroviral inhibitors exert on HIV. Broadly cross-reactive CTL responses have been associated with resistance to HIV infection and disease progression (77, 78), suggesting that pre-existing immunity against potential escape variants is key to long-term suppression of HIV replication.

To establish whether immune and drug selection pressures converge in HIV infection, we tested how drug resistance mutations within HIV CTL epitopes affected their recognition by CTL. More specifically, we examined whether there was increased, decreased or sustained recognition of CTL epitopes incorporating drug resistance mutations compared with wild-type epitopes and investigated the ability of HIV-specific CTL to differentiate between wild-type and variant epitopes. We further examined viral sequences from individuals demonstrating CTL recognition of drug resistant HIV to assess whether recognition of epitopes incorporating drug resistance mutations was associated with the presence of specific mutations that elicited CTL specific for variant epitopes containing drug resistance mutations.

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2. Antiretroviral Drug Resistance Mutations Sustain or Enhance CTL Recognition of Common HIV-1 *Pol* Epitopes

2.1. Abstract

Antiretroviral drug resistance and escape from CTL are major obstacles to effective control of HIV replication. To investigate the possibility of combining drug and immune-based selective pressures against HIV, we studied the effects of antiretroviral drug resistance mutations on CTL recognition of five HIV-1 Pol epitopes presented by common HLA molecules. We found that these common drug resistance mutations sustain or even enhance the antigenicity and immunogenicity of HIV-1 Pol CTL epitopes. Variable patterns of cross-reactive and selective recognition of wild-type and corresponding variant epitopes demonstrate a relatively diverse population of $CD8^+$ T cells reactive against these epitopes. Variant peptides with multiple drug resistance mutations still sustained CTL recognition, and some HIV-infected individuals demonstrated strong $CD8^+$ T cell responses against multiple CTL epitopes incorporating drug resistance mutations. Selective reactivity against variant peptides with drug resistance mutations reflected ongoing or previous exposure to the indicated drug, but was not dependent upon the predominance of the mutated sequence in endogenous virus. The frequency and diversity of CTL reactivity against the variant peptides incorporating drug resistance mutations and the ability of these peptides to activate and expand CTL precursors in vitro indicate a significant functional interface between the immune system and antiretroviral therapy. Thus, drug-resistant variants of HIV are susceptible to immune selective pressure that could be applied to combat transmission or emergence of

antiretroviral drug-resistant HIV strains and to enhance the immune response against HIV.

2.2. Introduction

The importance of $CD8^+$ T cells in the immune response against HIV has been clearly established. Anti-HIV CTL are often detectable in highly exposed individuals resistant to HIV infection, long term nonprogressors maintain strong, diverse anti-HIV CTL responses, and in acute infection, emergence of HIV-specific CTL precedes a sharp decline in viremia (1-6). Selection of CTL escape mutants parallels rising virus loads in HIV and SIV infections, and when $CD8^+$ T cell depletion before infection blunts CTL responses in SIV-infected monkeys, virus loads reach higher levels and remain high for a longer time (7-10). The immune system's disadvantage against a rapidly mutating virus is that it can only react to mutated peptide sequences after they emerge, whereas factors such as partial reactivity with altered peptide ligands and progressive immunodeficiency mitigate against efficient serial adaptation (11). Anticipating emerging mutants could reduce the disadvantage, but CTL escape mutations can vary widely within the agretope or epitope or even within flanking residues that affect protein processing (12, 13). Therefore, HIV escape from CTL can be relatively easily accomplished by various amino acid changes at loosely defined sites.

Escape of HIV from antiretroviral drugs is a major obstacle to effective long term antiretroviral therapy. Mutations conferring resistance within one or more of the three most extensively used antiretroviral drug classes, NRTIs, NNRTIs, and PIs, have become increasingly common in clinical HIV isolates (14). Unlike the mutations allowing escape from CTL, drug resistance mutations localize to particular genomic sites and conform to

specific amino acid changes. Those conferring NRTI and NNRTI resistance cluster within RT codons 41–236, whereas those conferring PI resistance cluster within PR codons 10–90. These regions of HIV-1 Pol also encompass a number of previously identified CTL epitopes (15). If these CTL epitopes remain immunogenic after incorporating drug resistance mutations, the well-documented constraints on drug resistance mutations could potentially be exploited to prime the immune system in anticipation of emerging mutants. Furthermore, if any epitopes incorporating drug resistance mutations exhibit enhanced antigenicity or immunogenicity, this would suggest that drug treatment itself could modulate the CTL response against HIV. Therefore, we investigated how a number of common antiretroviral drug resistance mutations in HIV *pol* affect CTL recognition and how the relative reactivity of CTL against wild-type Pol peptides and their corresponding variants incorporating drug resistance mutations relate to treatment history and predominant endogenous viral sequences.

2.3. Subjects and Methods

2.3.1. Study population

Our study cohort consisted of n=98 HIV-infected individuals attending the St. John's General Hospital HIV Clinic (St. John's, Newfoundland, Canada). HIV genotyping on selected plasma samples was conducted through a commercial service at the British Columbia Center for Excellence in HIV/ AIDS by reverse transcriptase – polymerase chain reaction (RT-PCR) amplification and automated sequencing of the entire PR-encoding and first 400 codons of the RT-encoding HIV-1 *pol* gene segments (www.hivresistanceweb.com). Informed consent was obtained for blood collection and

access to antiretroviral treatment records. This study was given ethical approval by the Memorial University Faculty of Medicine Human Investigation Committee.

2.3.2. Lymphocyte isolation and cell culture

Whole blood was collected by venipuncture into acid-citrate-dextrose-treated Vacutainers (BD Labware, Franklin Lakes, NJ) and peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice with phosphate buffered saline (PBS) plus 1% fetal calf serum (FCS) and resuspended in medium consisting of RPMI 1640 with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM N-2-Hydroxyethyl-piperazine-N'-2-Ethanesulfonic Acid (HEPES) buffer solution, and 2 X 10^{-5} M 2-mercaptoethanol (ME) (all from Life Technologies Invitrogen, Grand Island, NY). HLA class I-A and B type donors were determined as previously described (16) using commercial kits (One λ; Canoga, CA). Autologous B lymphoblastoid cell lines (BLCL) were generated by Epstein Barr virus (EBV) transformation of peripheral blood B cells. Briefly, 2.5 ml of supernatant from marmoset B95-8 leukocytes (CRL 1612; American Type Culture Collection, Manassas, VA) was passed through a 0.45-μm pore size sterile filter (Millipore, Bedford, MA) and added to 5 X 10^6 freshly isolated PBMC. These cells were then cultured for 24 h, washed, and maintained in medium with 20% FCS and 1 μg/ml cyclosporin A until sufficient growth occurred to cryopreserve several aliquots of the cell line.

2.3.3. Bulk anti-HIV CTL

To activate bulk anti-HIV CTL, 1/10th of an aliquot of freshly isolated PBMC was stimulated with 5 μg/ml purified phytohemagglutinin (PHA) (ICN Biomedicals, Aurora, OH) and 5 U/ml rIL-2 (Hoffmann-LaRoche, Nutley, NJ) for 3 days, washed twice with PBS plus 1% FCS, and added to the remaining cells in medium. After 3-day coculture, 5 U/ml IL-2 was added, and after a further 7-day expansion, cells were tested for anti-HIV CTL.

2.3.4. Enzyme-linked immunospot assay (ELISPOT) assay for IFN-γ release

Microtiter assay plates (Multiscreen; Millipore) were coated with 100 μl of 15 μg/ml anti-interferon-gamma (IFN-γ) monoclonal antibody (mAb) 1-D1K (Mabtech, Stockholm, Sweden) overnight at 4°C. The plates were then washed six times with PBS, and PBMC were added in duplicate at 2 X 10^5 cells/well in medium together with 0.4 μg/ml of the relevant peptide and incubated overnight. Negative control wells containing PBMC alone and PBMC plus control peptides known to bind to the HLA class I-A or B molecules presenting the test peptides were included. Positive control wells contained 4 μg/ml PHA. All peptides were >95% pure (Genemed Synthesis, San Francisco, CA). After overnight incubation, plates were washed as described above, and 100 μl/well of 1 μg/ml biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added for 2 h. Wells were washed six more times, and 100 μl of streptavidin-alkaline phosphatase conjugate (Mabtech) diluted 1/1000 was added for 1 h. Plates were again washed six times, and 100 μl chromogenic alkaline phosphatase substrate (Bio-Rad, Hercules, CA) diluted 1/10 in Tris HCl buffer, pH 9.5, was added. After 30 min, plates were washed with tap water to terminate color reactions and then air-dried. Spots were counted with an automated

ELISPOT counter (Zellnet Consulting, New York, NY). Responses were considered positive if the number of spots was more than twice the negative control and >50/10^6 PBMC.

2.3.5. Peptide-specific CTL stimulation

Peptide-specific CTL were generated *in vitro* as previously described (17). Briefly, 5 X 10^6 PBMC were pulsed with 100 μM peptide in 100 μl of medium for 1 h at 37°C. The cells were then resuspended at 2.5 X 10^6 cells/ml in medium supplemented with 25 ng/ml recombinant human IL-7 (R&D Systems, Minneapolis, MN). On day 3, 10 U/ml IL-2 was added, and cells were tested between days 10 and 14 for lysis of peptide-pulsed BLCL.

2.3.6. CTL assay

Autologous BLCL were used as targets. To screen for anti-HIV Pol CTL, BLCL were infected for 16 h with 10 PFU/cell of a recombinant vaccinia virus, vCF21, expressing the entire HIV-1 HXB2 *pol* gene, or a control vaccinia virus, vSC8, expressing *Escherichia coli* β-galactosidase (18). Both viruses were from the National Institutes of Health AIDS Research Reference Reagent Program (Bethesda, MD). Infected cells were washed and incubated in 100 μl of medium with 100 μCi of Na2 51CrO4 (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 37°C. Labeled cells were washed three times and transferred to U-bottom, 96-well plates (Corning Glass, Corning, NY) at 5 X 10^3 cells/well. To test peptide-specific recognition, individual peptides were added in duplicate at a final concentration of 20 μg/ml for 1 h at 37°C to 5 X 10^3 uninfected labeled target cells in 100 μl of medium. Effector cells were added to the desired effector-

to-target (E:T) cell ratios, and volumes were adjusted to 300 µl/well. Assays ran for 5 h, after which 125 µl of supernatant/well was counted in a Wallac 1280 gamma counter (Gaithersburg, MD). Specific lysis was calculated as: (experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximal ^{51}Cr release - spontaneous ^{51}Cr release) X 100. Spontaneous ^{51}Cr release was <15% in all assays reported.

2.4. Results

2.4.1. Selection of HIV-1 Pol CTL epitopes encompassing drug resistance mutations

By overlaying common, well-defined antiretroviral drug resistance mutations onto HIV CTL epitope maps, we found 11 previously known HIV Pol CTL epitopes in which drug resistance mutations occur (14). We focused on five epitopes restricted by the common HLA molecules A2, A3, B35, and B44 (Fig. 2.1). More than 90% of the subjects in our cohort expressed at least one of these common HLA class I alleles and many expressed two or more in combination. Therefore, these were the most practical epitopes with which to test the effects of the drug resistance mutations on immune recognition in our study cohort, and the results are applicable to the HIV-infected population in general. The different drug resistance mutations incorporated into the variant peptides we selected for synthesis and testing collectively reflect the impact of multiple PIs, NRTIs, and NNRTIs.

2.4.2. Recognition of peptides incorporating drug resistance mutations by bulk-cultured anti-HIV CTL

As the selected peptides all derive from HIV-1 Pol, study subjects were initially tested for Pol-specific CTL using vCF21, a recombinant vaccinia virus expressing the entire HIV-1 clade B HXB2 Pol protein. We detected CTL against HXB2 Pol in >60% of

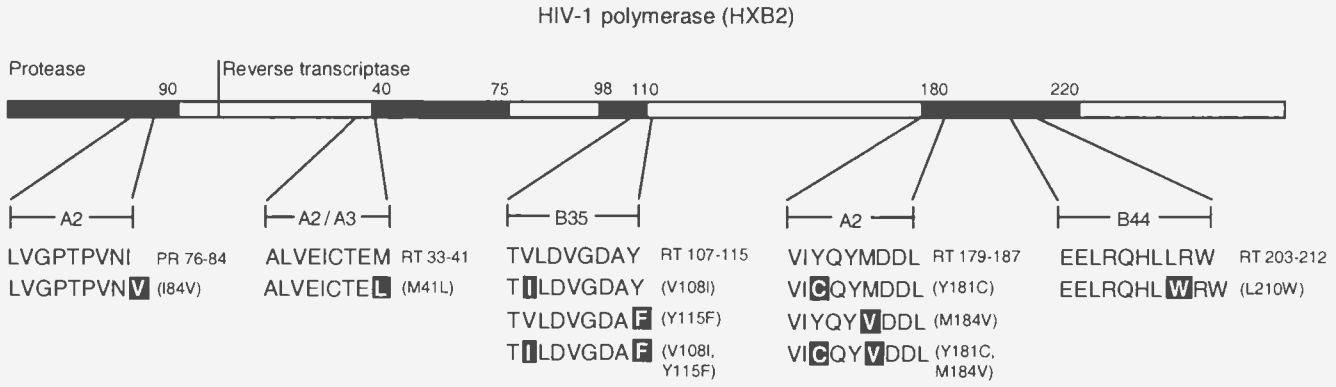


Figure 2.1

Schematic representation of regions of the HIV-1 *pol* gene encoding known CTL epitopes that overlap with common drug resistance mutation sites. The HLA restriction of each epitope is indicated above the wild-type (*top*) sequence. Shaded segments of the *pol* gene indicate regions where most of the drug resistance mutations cluster. Shaded amino acids indicate mutations commonly selected for by antiretroviral drugs. These mutations were incorporated into synthetic variant peptides and tested for recognition.

individuals tested (data not shown). Although recognition of epitopes containing drug resistance mutations need not correspond to recognition of this wild-type HIV laboratory strain Pol protein, peptide-specific CTL responses against our selected wild-type or variant Pol peptides were only detected in donors with Pol-specific CTL apparent using vCF21.

When bulk-stimulated anti-HIV CTL from n=98 donors with the appropriate HLA were tested against peptide-pulsed BLCL, three of five wild-type peptides and variants of four wild-type peptides triggered specific cytolysis. Wild-type peptides RT33–41 (Fig. 2.2*a*), RT107–115 (Fig. 2.2*b*), and RT203–212 (Fig. 2.2*d*) all triggered cytolysis by anti-HIV CTL. Variant peptides incorporating one of the M41L, V108I, Y115F, Y181C, or L210W mutations also triggered cytolysis (Fig. 2.2). Variant peptides incorporating two mutations, V108I and Y115F (Fig. 2.2*b*) or Y181C and M184V (Fig. 2.2*c*), also triggered cytolysis. Thus, common antiretroviral drug resistance mutations within the four CTL epitopes, RT33–41, RT107–115, RT179–187, and RT203–212, presented by HLA-A3, B35, A2, and B44, respectively, sustain the antigenicity of these epitopes for HIV-specific CTL. For RT107–115, recognition of wild-type and variant epitopes was cross-reactive at the bulk CTL level (Fig. 2.2*b*). For the RT33–41 and RT203–212 epitopes, there were instances of cross-reactive recognition of wild-type and variant peptides (Fig. 2.2*a* and *d,* subjects 121 and 131) and selective recognition of either the wild-type or variant peptide (Fig. 2.2*a* and *d,* subjects 012, 055, 057, 077, 082, and 126). For the RT179–187 epitope, selective recognition of variant peptides incorporating the Y181C (Fig. 2.2*c*, donor 028) or Y181C and M184V (Fig. 2.2*c*, donor 001) mutations occurred. These data indicate a complex CTL repertoire capable of recognizing HIV-1 Pol epitope

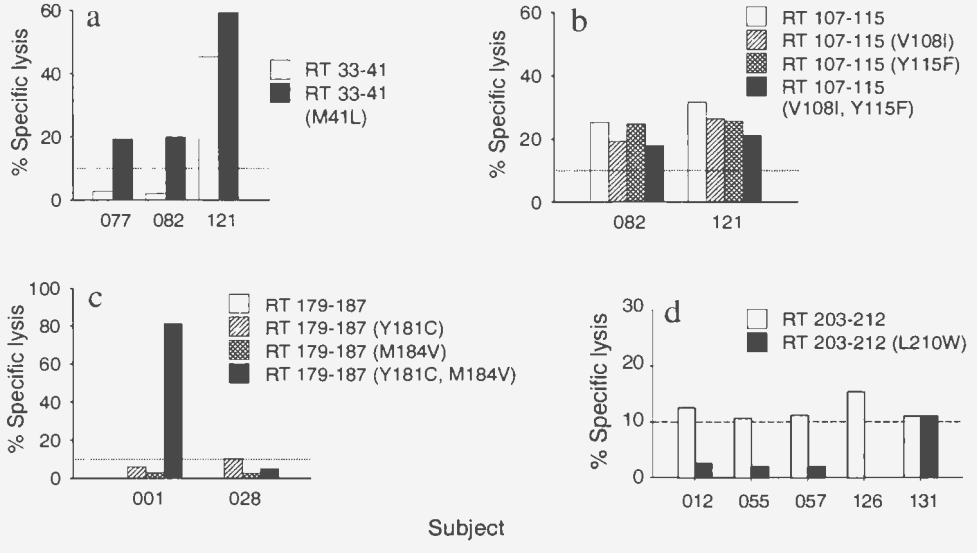


Figure 2.2

Recognition of wild-type Pol peptides and variants incorporating drug resistance mutations by bulk-cultured anti-HIV CTL. Lysis triggered by wild-type peptides RT33–41 (*a*), RT107–115 (*b*), RT179–187 (*c*), and RT203–212 (*d*) is shown with unshaded bars. Results with variant peptides are shown by hatched or dark bars, with specific mutations indicated in the legend. All results shown are for an E:T cell ratio of 50:1 with background lysis subtracted. The dashed line at 10% specific lysis represents the lower limit for a positive response.

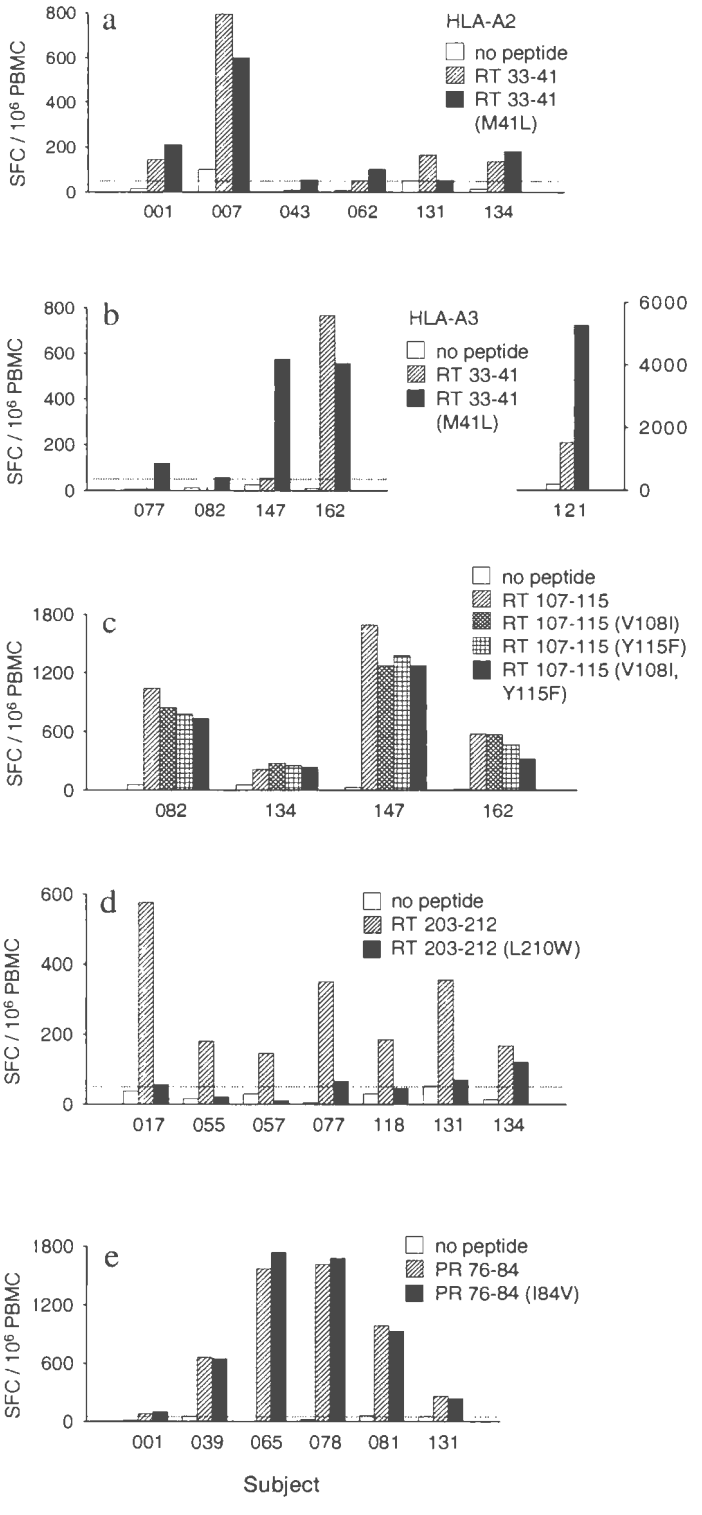
variants incorporating common drug resistance mutations and, in some cases, of distinguishing the variants from their wild-type counterparts.

2.4.3. IFN-γ production by PBMC exposed to peptides incorporating drug resistance mutations

As IFN-γ ELISPOT assays are sensitive and can estimate the frequency of peptide-specific T cells in unmanipulated PBMC, we tested freshly isolated or cryopreserved PBMC by ELISPOT for recognition of HIV-1 Pol CTL epitopes incorporating drug resistance mutations. The same three wild-type peptides and corresponding variants that triggered cytolysis by bulk-stimulated anti-HIV CTL stimulated IFN-γ-release from PBMC and recognition of the RT33–41 wild-type and M41L variant was detected in the context of HLA-A2 (Fig. 2.3*a–d*). In addition, both the PR76–84 wildtype epitope and I84V drug resistance mutation variant stimulated IFN-γ release (Fig. 2.3*e*). ELISPOT recognition patterns with PBMC were similar to those observed in lytic assays with bulk-stimulated anti-HIV CTL. Recognition of RT107–115 and PR76–84 wild-type epitopes and their corresponding drug resistance variants was cross-reactive (Fig. 2.3, *c* and *e*), whereas RT33–41 and RT203–212 wild-type and drug resistance variant peptides showed cross-reactive (Fig. 2.3*a,* subjects 001, 007, 062, and 134; Fig. 2.3*b*, subjects 121 and 162; Fig. 2.3*d*, subjects 077 and 134) and selective recognition (Fig. 2.3*a,* subjects 043, and 131; Fig. 2.3*b*, subjects 077, 082, and 147; Fig. 2.3*d,* subjects 017, 055, 057, 118, and 131). Cross-reactivity of the RT107–115 and PR76–84 wild-type and variant peptide epitopes was confirmed across a range of peptide concentrations from 4 μM to 400 pM (Fig. 2.4*a* and *b*). These ELISPOT data demonstrate a complex circulating T cell repertoire with CTL precursors capable of recognizing

Figure 2.3

Peptide-specific stimulation of IFN-γ release in PBMC from HIV-infected individuals by wild-type Pol peptides and variants incorporating drug resistance mutations. Unshaded bars show background spot-forming cells (SFC). The number of SFC triggered by wild-type and corresponding variant peptides RT33–41 (*a* and *b*), RT107–115 (*c*), RT203–212 (*d*), and PR76–84 (*e*) are shown with hatched or dark bars, with specific mutations indicated in the legend. The dashed line at 50 SFC/10^6 PBMC represents the lower limit for a positive response.


a
HLA-A2
no peptide
RT 33-41
RT 33-41 (M41L)
SFC / 10⁶ PBMC
800
600
400
200
0
001
007
043
062
131
134
b
HLA-A3
no peptide
RT 33-41
RT 33-41 (M41L)
6000
4000
2000
0
077
082
147
162
121
c
no peptide
RT 107-115
RT 107-115 (V108I)
RT 107-115 (Y115F)
RT 107-115 (V108I, Y115F)
1800
1200
600
082
134
147
162
d
no peptide
RT 203-212
RT 203-212 (L210W)
017
055
057
077
118
131
134
e
no peptide
PR 76-84
PR 76-84 (I84V)
001
039
065
078
081
131
Subject

HIV-1 Pol epitope variants incorporating common drug resistance mutations and, in some cases, distinguishing them from their wild-type counterparts. ELISPOT assays were repeated at several time points for most individuals. Although the absolute number of spot-forming cells changed over time, the reactivity patterns for the wild-type and variant peptides remained consistent, and representative data are shown.

The ELISPOT data clearly demonstrated recognition of peptides incorporating drug resistance mutations by circulating T cells from HIV-infected individuals. The hierarchy of epitopes recognized and the pattern of cross-reactivity between wild-type and corresponding variant peptides reflected those seen with bulk-cultured anti-HIV CTL. The peptide-specific T cell frequencies observed illustrate the antigenicity and suggest *in vivo* immunogenicity of variant peptides incorporating drug resistance mutations. Notably, the frequency of T cells producing IFN-γ in response to the RT33–41(M41L) variant peptide presented in the context of HLA-A3 reached ~1/200 PBMC, roughly 1% of circulating $CD8^+$ T cells (Fig. 2.3*b*). Most responding cells in this case did not cross-react with wild-type RT33–41, suggesting selective *in vivo* induction of high frequency CTL against viral variants incorporating the M41L drug resistance mutation.

2.4.4. Relationship among CTL specificity, treatment history, and HIV genotype

HIV genotypic information was obtained from each subject with plasma virus load >1000 RNA copies/ml to assess the relationship between predominant endogenous viral sequences at the time of testing, treatment history or current status, and specificity of anti-HIV CTL for wild-type or variant peptides. In some cases, genotypic information could not be obtained at all or could not be obtained coincident with immunological testing

because of undetectable plasma virus loads, but information was always taken from the closest feasible time point. Complete antiretroviral treatment history was available for all study participants. Relevant genotypic and treatment history information is summarized in Table 2.1. As there was CTL cross-reactivity for the PR76–84 and RT107–115 wild-type and variant peptides over a range of peptide concentrations (Fig. 2.4), neither the predominant endogenous sequence nor the treatment history can impact on CTL specificity. However, there could be *in vivo* selection for or against different endogenous variants by the CTL. Sequence information was only available for subject 082 with CTL against RT107–115 wild-type and variant peptides associated with NNRTI and abacavir (ABC) resistance. Although he was receiving the NNRTI sustiva and had a high plasma virus load, no mutations associated with NNRTI or ABC resistance were detected within RT107–115 at the time the CTL assay shown in Fig. 2.2*b* was performed.

Sequence information was available for subjects 001, 065, and 078 with CTL against PR76–84. All were receiving PIs at the time of testing, and the I84V mutation was predominant in subjects 065 and 078. This shows that the HLA-A2-restricted PR76–84 peptide with or without the drug resistance-associated mutation I84V can persist in endogenous virus in the face of a specific CTL response. The genotype for endogenous virus of subjects 001 and 065 was obtained from the same time point as the ELISPOT data, whereas the closest sample from subject 078 with a detectable plasma virus load was taken 18 months before the ELISPOT.

As $CD8^+$ T cells responding to RT33–41, RT179–187, and RT203–212 were only partially cross-reactive with wild-type and variant peptides, we expected to see some impact of treatment history and predominant endogenous viral sequence on relative levels

Table 2.1. Treatment History and Endogenous Viral Sequence of Individuals with $CD8^+$ T cell Recognition of Epitopes Incorporating Antiretroviral Drug Resistance Mutations

Subject	Recent Treatment History	HIV genotype
001	DDI, 3TC, ABC, amprenavir (10/00-present)	RT M41L, M184V
	DDI, d4T, sustiva, ritonavir, indinavir (6/99-10/00)	RT M41L
012	d4T, 3TC, sustiva (9/99-present)	RT L210
017	d4T, 3TC (11/98-present)	Virus load too low
028	3TC, sustiva, nelfinavir (6/99-present)	Virus load too low
039	AZT, 3TC, ritonavir, saquinavir (11/00-present)	Virus load too low
043	d4T, 3TC, DDC (2/99-present)	Virus load too low
055	sustiva, saquinavir, ritonavir (5/00-present)	Virus load too low
	ABC, saquinavir, ritonavir (1/99-5/00)	
057	Nil (7/01-present) 3TC, d4T saquinavir (8/97-7/01)	PR I84V
062	DDI, d4T, sustiva (7/01-present)	RT M41L
065	3TC, ABC, sustiva (8/00-present)	PR I84V
	d4T, DDI, indinavir (3/98-8/00)	
077	Nil (7/02-present), 3TC, d4T (5/01-7/02)	RT M41, L210
078	3TC, d4T, kaletra (3/02-present) Nil (5/00-3/02)	PR I84
081	DDI, d4T, ritonavir, saquinavir (9/99-present)	Virus load too low
082	DDI, ABC, kaletra (2/02-present)	RT M41, V108, Y115
	DDI, d4T sustiva (7/99-2/02)	
118	AZT, 3TC, indinavir (4/99-present)	Virus load too low
121	AZT, 3TC, ABC 0/02-present)	Virus load too low
	Nevirapine, 3TC, ABC (6/00- 0/02)	
126	AZT, 3TC, sustiva (5/99-present)	Virus load too low
131	3TC, d4T, sustiva (6/00-present)	Virus load too low
134	d4T, 3TC, sustiva (10/99-present)	Virus load too low
147	3TC, ABC, sustiva (5/00-present)	Virus load too low
162	AZT, 3TC, sustiva (4/02-present)	RT M41, V108, Y115

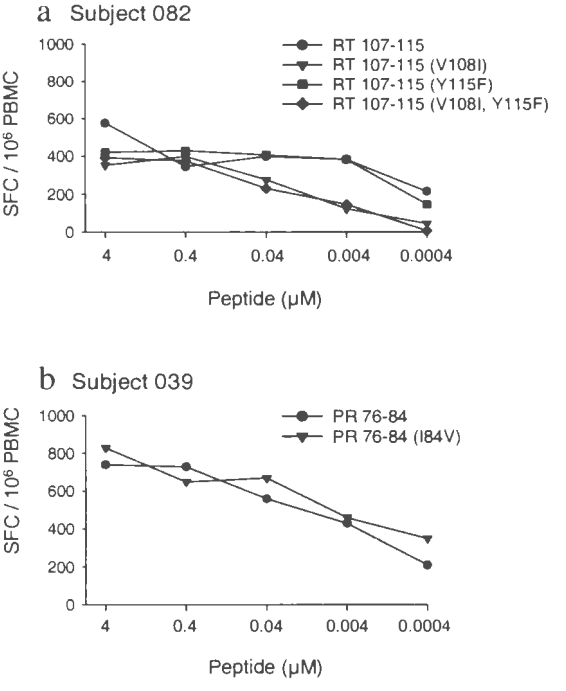


Figure 2.4

Cross-reactivity of CD8+ T cells with RT107–115 and PR76–84 wild-type and corresponding peptides incorporating drug resistance mutations at varying peptide concentrations. PBMC from subject 082 (HLA-B35) and 039 (HLA-A2) were tested by ELISPOT for reactivity against RT107–115 (*a*) and PR76–84 (*b*) wild-type and variant peptides across a range of peptide concentrations from 4 μM to 400 pM.

of reactivity of these CTL against wild-type and variant peptides. The RT33–41 wild-type and M41L variant peptides were recognized in the context of 6 HLA-A2 subjects (Fig. 2.3*a*) and in the context of 5 HLA-A3 subjects (Figs. 2.2*a* and 2.3*b*) with differential skewing toward either wild-type or mutant peptide.

Recognition of the wild-type RT33–41 peptide and M41L variant associated with azidothymidine (AZT) and stavudine (d4T) resistance was mixed for the six HLA-A2 individuals (Fig. 2.3*a*). Subject 001 was receiving abacavir (ABC), and the rest were receiving d4T at the time of testing. Although ABC does not select for the M41L mutation, once acquired this mutation can contribute to ABC resistance (www.hivresistanceweb.com). Subject 001 had previously been treated with d4T and AZT and acquired the M41L mutation before ABC treatment. Sequence data coincident with the ELISPOT results were available for 001 and 062, both of whom had predominant M41L mutant endogenous virus. Sequence data from subject 007 could only be obtained from a plasma sample taken 18 mo before the ELISPOT, which showed predominantly M41 wild-type virus. These results suggest that exposure to AZT or d4T sensitizes $CD8^+$ T cells from HLA-A2 individuals against the RT33–41 peptide epitope containing the M41L resistance-associated mutation and show that the epitope containing the M41L mutation persists in the face of this HLA-A2-restricted immune response.

Recognition of the variant peptide incorporating the M41L mutation was predominant for four of the five HLA-A3 subjects (Fig. 2.3*b*). One subject (121) was receiving AZT at the time of testing. The others were receiving d4T (077), AZT (162), or ABC (082 and 147) at the time of testing. Subject 082 had been receiving d4T until within 1 month of the CTL assay shown in Fig. 2.2*a* and until within 6 months of the

ELISPOT assay. Subject 147 had received AZT and d4T in the past, but not within 18 months of the ELISPOT assay. Endogenous viral sequence data for subject 082 revealed wild-type RT33–41 as the predominant endogenous species. Subject 162, who was the only HLA-A3 subject to preferentially recognize the wild-type sequence, had started antiretroviral therapy with AZT, lamivudine (3TC), and sustiva only 1 month before testing. His predominant endogenous viral sequence was also wild-type. These results suggest that exposure to AZT or d4T sensitizes HIV-infected HLA-A3 individuals against the RT33–41 peptide epitope containing the M41L resistance-associated mutation. This sensitization persists without predominance of the mutation in endogenous virus. In contrast to the HLA-A2 subjects, the M41L mutation was not seen in any of the three HLA-A3 individuals with a $CD8^+$ T cell response against the RT33–41 M41L variant epitope for whom genotypic information was available.

With the RT179–187 epitope, we saw exclusive recognition of peptides incorporating resistance mutations by two individuals (Fig. 2.2*c*). Subject 028, who was receiving the NNRTI sustiva, recognized a peptide with NNRTI-associated mutation Y181C. Subject 001, who was receiving the NRTIs didanosine, lamivudine, and ABC, recognized a peptide with both Y181C and M184V mutations. The M184V mutation is associated with 3TC, didanosine (DDI), zalcitabine (DDC), and ABC resistance. His predominant endogenous virus at the time incorporated the M184V mutation, but not Y181C, and he had not received NNRTIs within 4 years of testing.

All seven individuals who recognized the RT203–212 epitope preferentially recognized the wild-type epitope over that incorporating the L210W mutation (Fig. 2.3*d*). The L210W mutation, like M41L, is selected for by AZT and d4T, but can also contribute

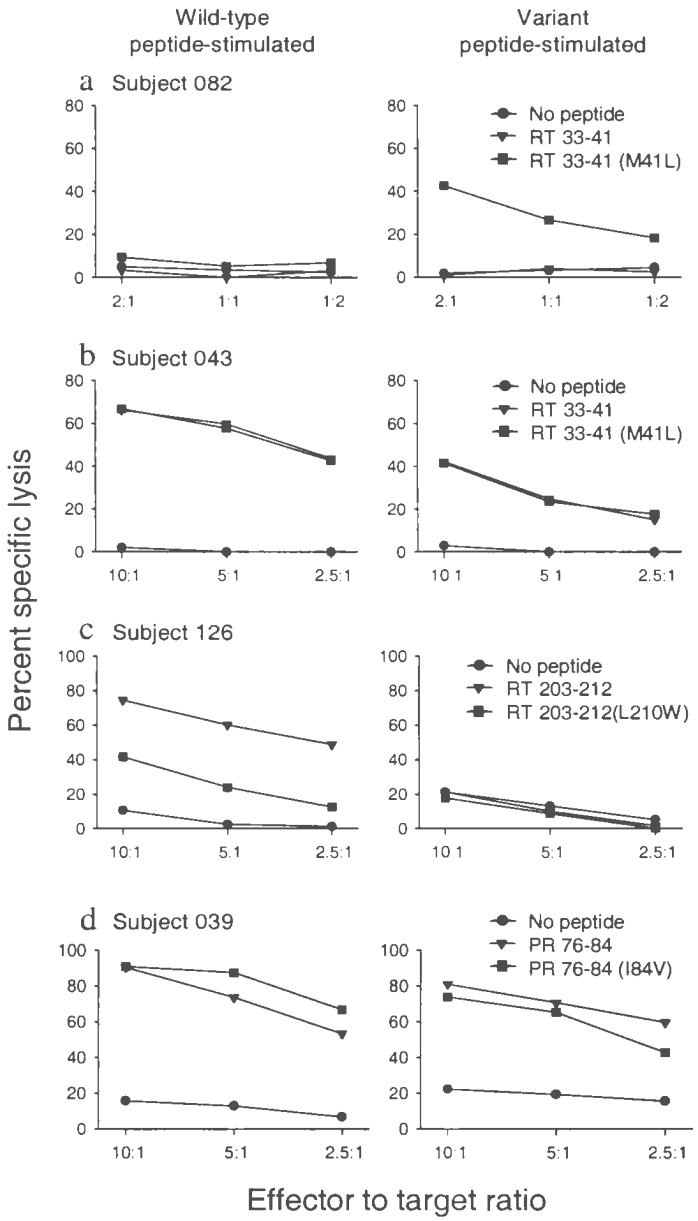
to ABC resistance, once established. Both subjects (077 and 134) who showed some recognition of the mutant epitope were receiving d4T. Two individuals who exclusively recognized the wild-type epitope had either never received AZT or d4T (055) or had not received either of these drugs for at least 2 years (057). The endogenous viral sequence from subject 057 from a sample taken nearly 1 year after the ELISPOT assay was predominantly wild-type within RT203–212. Three other HLA-B44 individuals who exclusively recognized the wild-type epitope were nonetheless receiving d4T (017 and 131) or AZT (118). Thus, it appears that exposure to AZT or d4T can sensitize HLA-B44 individuals to a variant peptide epitope incorporating the L210W mutation, but that this variant is less immunogenic than the wild-type epitope.

2.4.5. Peptide-specific CTL stimulation

The ability of wild-type and variant peptides to activate CTL *in vitro* was tested by peptide-specific PBMC stimulation. Although all peptides were antigenic, the pattern of *in vitro* activation of self-reactive versus cross-reactive CTL varied for individual peptides (Fig. 2.5). For HLA-A3$^+$ subject 082, the RT33–41 M41L variant activated CTL specific for itself without cross-reactivity for the wild-type RT33–41 peptide, whereas the wild-type peptide activated no CTL against itself or the variant (Fig. 2.5*a*). This reflected the selective recognition of the variant peptide that we observed by bulk cytotoxicity and ELISPOT (Figs. 2.2*a* and 2.3*b*). Subject 162 showed the same response pattern as 082 after re-stimulation (data not shown), which did not match the ELISPOT data showing preferential recognition of the wild-type peptide, but did match the apparently enhanced *in vitro* immunogenicity of the M41L variant overall. In the

Figure 2.5

Specificity of CTL induced by PBMC stimulation with wild-type Pol peptides or variants incorporating drug resistance mutations for wild-type Pol peptides or variants. The *left graph* shows results for stimulation with wild-type peptides and variant peptide stimulation on the right. Peptides tested for recognition are shown to the *right graph* of each series. Representative specificity or cross-reactivity patterns for the RT33–41 (*a* and *b*), RT203–212 (*c*), and PR76–84 (d) wild-type and variant peptides are shown.


Wild-type peptide-stimulated
Variant peptide-stimulated
a Subject 082
b Subject 043
c Subject 126
d Subject 039
No peptide
RT 33-41
RT 33-41 (M41L)
RT 203-212
RT 203-212(L210W)
PR 76-84
PR 76-84 (I84V)
Percent specific lysis
Effector to target ratio

context of HLA-A2, both the wild-type and variant peptides stimulated CTL from subjects 043 (Fig. 2.5*b*), 001, 007, and 134 (data not shown), who were cross-reactive for both peptides. This matches the generally cross-reactive recognition of both the wild-type and variant peptides seen in the ELISPOT assays. For subjects 126 (Fig. 2.5*c*) 017, 055, and 131 (data not shown), the wild-type RT203–212 peptide activated CTL cross-reactive against itself and the L210W variant peptide, whereas the L210W variant did not activate CTL against either peptide. This matched the ELISPOT data in that reactivity against the wild-type peptide was favored, but the wild-type peptide stimulated CTL against the L210 variant even in subjects who showed no significant reactivity with the L210W variant peptide by ELISPOT. For subject 039, both the wild-type PR76–84 and I84V variant peptide induced CTL cross-reactive against both peptides (Fig. 2.5*d*), matching the ELISPOT data showing complete cross-reactivity between the wild-type and I84V variant peptides. Overall, the peptide stimulation results indicate that for the HLA-A3-restricted RT33–41 and HLA-B44-restricted RT203–212 epitopes, the reactive T cell population in HIV-infected individuals includes T cells that distinguish between wild-type and variant peptides and T cells that cross-react with both wild-type epitopes and variants incorporating drug resistance mutations. For the HLA-A2-restricted RT33–41 and PR76–84 peptides, T cells appear to predominantly cross-react with the wild-type and variant peptides.

2.5. Discussion

In this study we examined the effect of antiretroviral drug resistance-associated mutations on recognition of HIV-1 Pol CTL epitopes presented by common HLA molecules. Although cross-reactivity patterns differed, variant peptides incorporating

common mutations associated with the three major antiretroviral drug classes all sustained and in some cases even enhanced T cell recognition. Recognition of the wild-type and variant peptides encompassing sites of common drug resistance mutations was quite prevalent in our study cohort with relatively high frequencies of reactive T cells. It was not uncommon for individuals to show T cell reactivity against multiple epitopes potentially or actually incorporating drug resistance mutations, indicating a strong immunological focus on functional determinants of the HIV Pol proteins.

We saw several patterns in terms of relative recognition and *in vitro* immunogenicity levels of wild-type and variant peptides that in most cases reflected antiretroviral treatment history. In the case of the HLA-A2-restricted PR76–84 and HLA-B35-restricted RT107–115 epitopes, recognition and immunogenicity patterns were independent of treatment history due to the complete cross-reactivity of the wild-type and variant peptides. The HLA-B44-restricted RT203–212 wild-type epitope was always preferentially recognized relative to the L210W variant, and the variant was only recognized by individuals who had been treated at some point with antiretrovirals known to select for the L210W mutation. Despite the apparent *in vivo* immunogenicity of the L210W variant peptide, it failed to expand CTL against itself *in vitro*, whereas with PBMC from individuals sensitized *in vivo* against the L210W peptide, the wild-type peptide expanded CTL against itself and the L210W variant. Although this might suggest immunological selection against the wild-type sequence in HLA-B44 individuals, we did not observe the L210W mutation in any of 3 HLA-B44 individuals for whom viral sequence data were available regardless of their ongoing or previous antiretroviral treatment.

The HLA-A3-restricted RT33–41 epitope exhibited the most interesting pattern of recognition and immunogenicity. Although there was some cross-reactivity, the M41L variant peptide was nearly always preferentially recognized by CTL or PBMC from HIV-infected individuals currently or previously receiving antiretrovirals selecting for the mutation. This preferential recognition pattern, sustained even in the absence of *in vivo* M41L predominance, suggests that the M41L mutation increases the immunogenicity of this epitope *in vivo*. *In vitro* activation and expansion of CTL reinforced this suggestion, as the M41L variant peptide activated CTL against itself, but not the wild-type peptide, whereas the wild-type peptide was non-immunogenic *in vitro*, even with PBMC from individuals preferentially recognizing the wild-type peptide. Preferential recognition and enhanced immunogenicity of RT33–41 incorporating the M41L mutation raise the possibility of immunological selection against this drug resistance-associated mutation *in vivo* in HLA-A3 individuals. Despite an overall high frequency of the M41L mutation, this mutation was only seen in two HLA-A3 individuals in our cohort, both of whom were severely immunodeficient with no residual $CD8^+$ T cell response against HIV detectable. This contrasts with the RT33–41 epitope presented in the context of HLA-A2, where recognition of both the wild-type peptide and the M41L variant is common, with no general preference observed, and both peptides activate and expand CTL against themselves and the corresponding variant *in vitro*. Consistent with this apparent relatively equal immunogenicity, the M41L mutation was seen in two of the HLA-A2 individuals with $CD8^+$ T cells against this epitope, suggesting no immunological selection against either variant. Longitudinal studies of a larger number of HLA-A2 and A3 individuals with $CD8^+$ T cell responses against RT33–41 could best evaluate

immunological selection against the M41L mutation associated with AZT and d4T resistance. Of note in this regard was the endogenous viral sequence of subject 082. Despite his preferential recognition of RT33–41 incorporating the M41L mutation, sequencing of endogenous virus at six historical time points spanning >6 years revealed no M41L mutations. However, over the last four time points covering 4 years, the T215Y mutation was present (data not shown). Together with the M41L mutation, T215Y or T215F confers high level resistance to AZT or d4T, and therefore, these mutations almost always occur in tandem (www.hivresistanceweb.com). Subject 082 was unique among 10 individuals with T215Y/F in our cohort in the absence of the complementary M41L mutation in the predominant endogenous virus. The M41L mutation never occurred in the absence of the complementary T215Y/F mutation. Although more studies are necessary to prove this point, these results suggest that the M41L mutation in the context of HLA-A3 can be immunogenic when present at levels below which it is detectable by commercial genotyping methods and that this immune response exerts selective pressure against accumulation of the mutation and emergence of high level AZT and d4T resistance.

Although the effects on immunogenicity of the variants are neutral, positive, or negative, respectively, the HLA-A2-restricted RT33–41, HLA-A3-restricted RT33–41, and HLA-B44-restricted RT203–212 drug resistance variant recognition patterns demonstrate a complex available T cell repertoire of $CD8^+$ T cells, some cross-reactive and some specific for particular variants. Previous studies have shown recognition of peptides incorporating drug resistance mutations that in at least some cases was specific for the variant incorporating the drug resistance mutation (19-21). Both cross-reactive

recognition and specific recognition of drug resistance associated variant peptides offer opportunities for exploitation of the available $CD8^+$ T cell repertoire, especially when the variant peptides have enhanced immunogenicity. For example, the general cross-reactivity of the HLA-A2-restricted RT33–41 and PR76–84 and the HLA-B35-restricted RT107–115 variants implies that these peptides could be used therapeutically to boost immunity against both wild-type virus and drug-resistant variants. Inclusion of such epitopes in prophylactic vaccines would potentially offer coverage against both wild-type HIV and the drug-resistant HIV strains now being transmitted with increasing frequency (22, 23). The partially selective recognition of other drug resistance-associated CTL epitopes offers different opportunities. Firstly, as the T cell populations recognizing the wild-type and variant peptides are not completely overlapping, it implies the variants could be used to both strengthen and diversify the CTL response against HIV. Diversification to include CTL responses selective for drug-resistant HIV might be especially effective if evoked before using antiretroviral drugs selecting mutations represented in the variant epitopes used for immunization. Pre-existing immunity against drug-resistant viruses present at low frequencies, if present at all, should delay the emergence of drug resistance. Another implication of selective recognition of M41L and L210W mutation-incorporating variants is an opportunity to strengthen and diversify the anti-HIV CTL response through novel forms of autoimmunization without treatment interruption. Both mutations reflect the selective impact of AZT and d4T; therefore, manipulating the relative dominance of endogenous viral variants by cyclical administration and withdrawal of one or both of these drugs could augment immunity against both wild-type and drug-resistant HIV.

Documenting the antigenicity and immunogenicity of CTL epitopes incorporating antiretroviral drug resistance mutations is a first step toward developing immune strategies against the emergence and transmission of drug-resistant HIV. Although the immunogenicity of these epitopes is fundamental, a number of additional factors will determine the antiviral efficacy of CTL they might induce. Pol is a major T cell antigen in human HIV infection, and importantly, HLA-associated selection for sequence variation within Pol CTL epitopes demonstrates selective pressure of anti-HIV Pol CTL *in vivo* (24-26). In humans and macaques, CTL activity against just one epitope can sustain relative viral quiescence and slow disease progression (9, 10). The five CTL epitopes and corresponding drug resistance variants we tested are presented by HLA-A2, -A3, -B35, and -B44, four alleles that together cover >90% of people of western European descent and a majority of most other ethnic populations. Other CTL epitopes with drug resistance mutations are known, and more almost certainly remain to be identified (19-21). Thus, broadly applicable multiepitope vaccines against drug-resistant HIV are feasible.

2.6. Acknowledgments

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3. Cross-reactive CTL Against HIV-1 PR and IFN-γ-Inducible Protein 30

3.1. Abstract

The IFN-γ-inducible protein (IP)-30 signal peptide -11 to -3 (LLDVPTAAV) is a prominent self peptide expressed with the class I HLA-A2. Stimulation of PBMC from HLA-A2 HIV-1-infected individuals with an HLA-A2-restricted HIV protease (PR) peptide 76-84 (LVGPTPVNI) activated CTL against the IP-30 signal peptide. Since HIV-1 PR 76-84 stimulated $CD8^+$ T cells from these individuals to secrete IFN-γ, we tested whether the activation of IP-30-specific CTL *in vitro* resulted from T cell cross-reactivity or from up-regulation of IP-30 by IFN-γ. Neither high levels of exogenous IFN-γ, nor incubation of PBMC with other HIV peptides triggering substantial IFN-γ release activated IP-30 specific CTL. Although the IP-30 signal peptide did not stimulate IFN-γ release from freshly-isolated PBMC, it activated CTL *in vitro* against itself and HIV PR 76-84. Peptide- stimulated IFN-γ release, cold target inhibition, and HLA-A2/Ig dimer-mediated binding and depletion of effector cells all indicated that *in vitro* stimulation with HIV PR 76-84 or the IP-30 signal peptide activated a comparable population of cross-reactive effector cells. Neither IP-30 nor HIV PR 76-84 activated CTL against themselves following *in vitro* stimulation of PBMC from non-HIV-infected HLA-A2 individuals. Peptide titrations indicated higher avidity T cell interactions with HIV PR 76-84 than with the IP-30 signal peptide. These data indicate that HIV PR76-84 is a heteroclitic variant of the IP-30 signal peptide -11 to -3, which has implications for immune memory and autoimmunity.

3.2. Introduction

Viral infections can trigger immunopathology or autoimmunity through a variety of mechanisms. Immunopathology results when the antiviral immune response targeting infected or otherwise sensitized host cells damages self tissues, such as in viral hepatitis. Autoimmunity results from the selective activation and expansion of normally quiescent self-reactive B or T lymphocytes. Molecular mimicry between viral and self-proteins, selective up-regulation of certain host proteins, virus-induced changes in the processing of host-proteins, enhanced antigen presenting cell (APC) function and pro-inflammatory environmental conditioning can all facilitate induction of autoimmunity or immunopathology by viral infections (1-8). While even acute infections can trigger immunopathology and autoimmunity, the persistent influence of chronic viral infections increases the probability of these immunological side-effects.

HIV-1 establishes chronic infection in essentially 100% of cases and has been associated with both immunopathology and autoimmunity. Immunopathology related to the pronounced $CD8^+$ T cell activation characteristic of HIV infection includes skin rashes, alveolitis, sicca-syndrome, lymphadenopathy, neuritis and vasculitis (9-14). Autoreactive $CD8^+$ T cells specific for self-peptides selectively overexpressed in HIV-infected cells have also been identified in HIV-infected individuals (15,16). In this context, both the pro-immune cytokine IFN-γ itself and a number of IFN-γ-responsive genes are expressed at elevated levels in lymphatic tissue when HIV replication is not fully suppressed (17). This alters the protein expression pattern and enhances the antigen-

presenting capacity of IFN-γ responsive cells, which could subsequently favor activation of cross-reactive T cells against IFN-γ-inducible proteins.

The signal peptide -11 to –3 (LLDVPTAAV) from an IFN-γ-inducible protein (IP) termed IP-30 is a dominant self-peptide expressed in the context of HLA-A2 (18). IP-30 was originally identified as an IFN-γ-inducible protein ubiquitously expressed in IFN-γ-stimulated PBMC and distributed in the lysosomes (19). More recently, IP-30 was shown to localize within MHC class II compartments (MIIC) (20) and therein catalyze reduction of disulfide bonds in proteins endocytosed by APC (21). Therefore, IP-30 is also referred to as γ-IFN-inducible lysosomal thiol reductase (GILT). Due to the central role of IP-30 in antigen processing and presentation and the prominent expression of the IP-30 signal peptide in the context of HLA-A2, we reasoned that T cell tolerance would be strictly enforced against the IP-30 signal peptide and chose it as a control HLA-A2 binding peptide for studying HIV-specific HLA-A2-restricted $CD8^+$ T cell responses. Although we never observed *ex vivo* IFN-γ release by $CD8^+$ T cells in response to the IP-30 signal peptide, CTL against HLA-A2 target cells pulsed with IP-30 signal peptide arose following *in vitro* stimulation of PBMC with the HIV PR peptide 76-84 (LVGPTPVNI). Since activation of IP-30-specific CTL was associated with strong *ex vivo* IFN-γ release in response to the PR 76-84 peptide and IFN-γ increases IP-30 expression levels, we tested whether *in vitro* induction of IP-30 signal peptide-specific CTL by HIV PR 76-84 reflected the impact of elevated IFN-γ levels or cross-reactivity between the 2 peptides. We found that in HIV-infected HLA-A2 individuals, the $CD8^+$ T cell response against HIV PR 76-84 included self-reactive $CD8^+$ T cells specific for the IP-30 signal peptide.

These cross-reactive CTL were readily activated *in vitro* by either HIV PR 76-84 or the IP-30 signal peptide itself. The IP-30 signal peptide also activated CTL *in vitro* against HIV PR 76-84. Such cross-reactivity may have both pathological potential, in the context of autoimmunity against APC, and adaptive potential in the context of an IFN-γ responsive self-peptide acting as a surrogate peptide supporting the maintenance or propagation of a select subset of anti-viral CTL.

3.3. Materials and Methods

3.3.1. Study population

Subjects for this study were a subset of HLA-A2 individuals nested within a study cohort of HIV-infected individuals attending the St. John's General Hospital HIV Clinic, St. John's, Newfoundland, Canada. Laboratory personnel expressing HLA-A2 served as non-HIV-infected controls. Informed consent was obtained for blood collection and this study was given ethical approval by the Memorial University Faculty of Medicine Human Investigation Committee. Blood was drawn concurrently for these studies and for standard clinical follow-up, which included plasma virus load measurement, blood chemistry, and lymphocyte subset analysis.

3.3.2. Lymphocyte isolation and cell culture

As outlined in section 2.3.2.

3.3.3. IFN-γ ELISPOT assay

As outlined in section 2.3.4.

3.3.4. Peptide-specific CTL stimulation

As outlined in section 2.3.5.

3.3.5. CTL assay

As outlined in section 2.3.6.

3.3.6. Intracellular flow cytometry

Stimulator cells were generated by incubating 1.0 X 10^5 BLCL for 1 hr with 20 μM peptide at 37°C, 5% CO_2 for 1 h. BLCL incubated as above without peptide served as negative controls. Peptide-stimulated CTL were added to the stimulator cells at a 5:1 ratio and incubated 5 h at 37°C, 5% CO_2. Brefeldin A (Sigma-Aldrich, Oakville, ON, Canada) was added to cells for the final 4 h at 10 μg/ml. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer containing 5 mM ethylenediamine-tetraacetic acid (EDTA), 0.5% bovine serum albumin (BSA), 0.02% sodium azide (all from Sigma) in PBS, incubated with phycoerythrin (PE)-labeled anti-human CD8 (Caltag, Hornby, ON, Canada) at 4°C for 20 min and then washed again with FACS buffer. Cells were then fixed with Fixation Buffer A (DAKO Cytomation Inc., Mississauga, ON, Canada), permeabilized with Perm Buffer (DAKO) and incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-IFN-γ mAb (Caltag) at 4°C for 20 min. The cells were then washed, resuspended in 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer.

3.3.7. Flow cytometry and T cell depletion with HLA-A2:Ig dimer

Empty HLA-A2:Ig dimer protein (BD Biosciences Pharmingen, Mississauga, ON, Canada) was loaded with peptide as per manufacturer's instructions. Briefly, HLA-A2:Ig dimer was mixed with 160 M excess peptide in PBS, pH 7.2 and incubated overnight at 37°C. Lyophilized peptides were dissolved at 50 mg/ml in DMSO and diluted 1:25 in

sterile PBS, pH 7.2. Empty HLA-A2:Ig dimer served as a negative control. Peptide stimulated PBMC (CTL lines) were incubated at 1 X 10^6 cells per 50 µL FACS buffer with 8 µl FcR blocking reagent (Miltenyi Biotec, Auburn, CA) at 24°C for 10 min. After this, 2 µg peptide-loaded HLA-A2:Ig dimer was added and the cells were incubated at 4°C for 1 hour. Cells were then washed twice with FACS buffer followed by a second incubation with 8 µl FcR blocking reagent (Miltenyi) at 24°C for 10 min. PE-conjugated A85-1 mAb (rat anti-mouse IgG1) (BD Biosciences Pharmingen) was then added and cells were incubated at 4°C for 30 min. The cells were then washed twice in FACS buffer and resuspended in 1% paraformaldehyde for analysis on a FACSCalibur flow cytometer (BD Biosciences).

For depletion experiments, cells were incubated with peptide-loaded HLA-A2:Ig dimer as above, washed in PBS supplemented with 0.5% BSA and 2 mM EDTA, pH 7.2, and incubated with a 10:1 ratio of goat anti-mouse IgG-coated magnetic beads (Dynal Inc, Brown Deer, WI, USA) at 4°C for 45 min. Bead-bound cells were removed by magnetic attraction and the unbound cells tested for cytotoxicity against selected target cells.

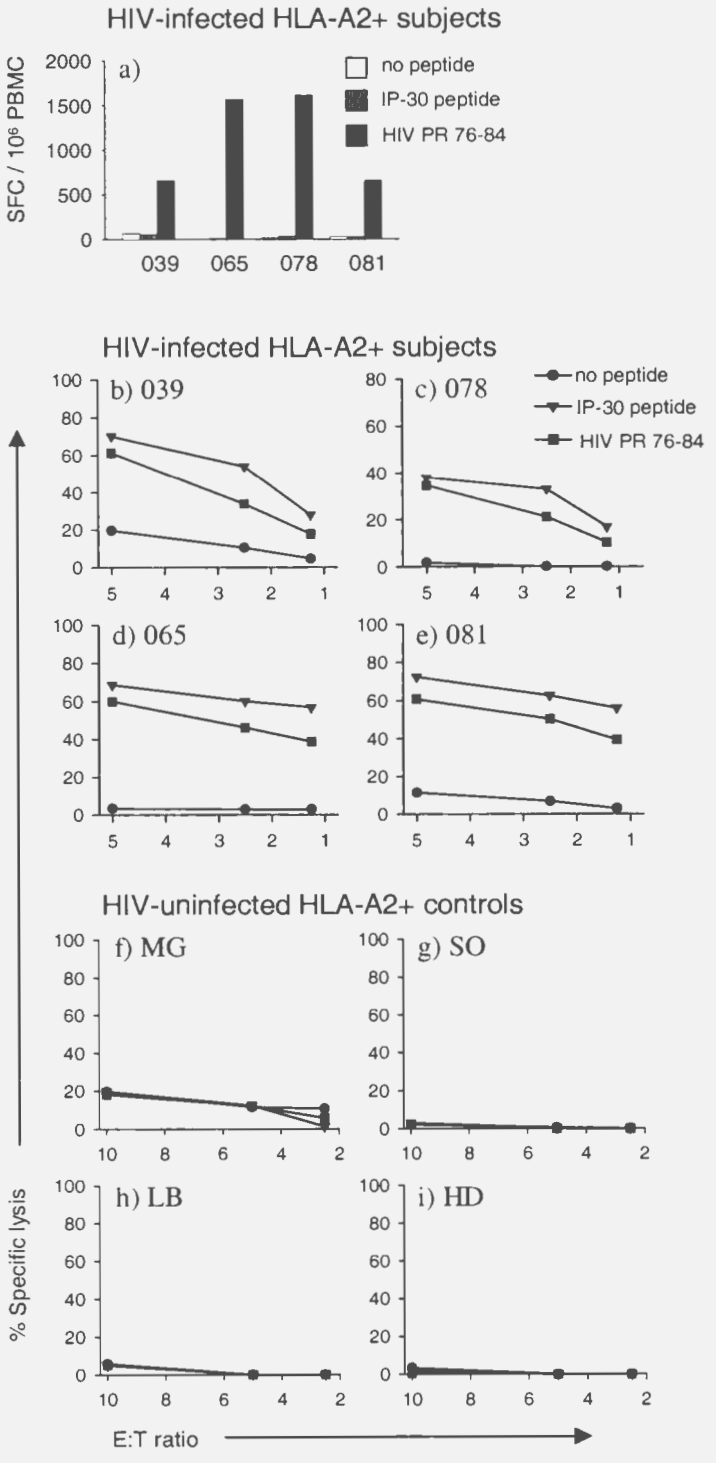
3.4. Results

3.4.1. HIV PR 76-84 activates CTL against the IP-30 signal sequence peptide in HIV-infected individuals expressing HLA-A2

We originally chose the HIV PR 76-84 peptide LVGPTPVNI for *in vitro* stimulation of CTL in order to study cross-reactivity between this peptide and a variant incorporating the I84V mutation associated with HIV protease inhibitor resistance (24).

Following *in vitro* stimulation with the HIV PR 76-84 peptide LVGPTPVNI, we observed specific lysis of HLA-A2 target cells pulsed with the IP-30 signal sequence peptide LLDVPTAAV in an individual with a high frequency of PBMC producing IFN-γ in response to HIV PR 76-84. Therefore, we tested 3 additional individuals with strong *ex vivo* PBMC IFN-γ responses to HIV PR 76-84 for CTL against IP-30 following *in vitro* stimulation with HIV PR 76-84 (Fig. 3.1 a). Although there was no demonstrable *ex vivo* IFN-γ release in response to the IP-30 signal sequence peptide in any case, even with 4-fold higher levels of peptide than normally used (data not shown), *in vitro* stimulation of PBMC from all 4 individuals with HIV PR 76-84 activated CTL against target cells pulsed with the IP-30 signal sequence peptide (Fig. 3.1 b-e). None of 6 non-HIV-infected HLA-A2 individuals generated CTL against the IP-30 signal sequence peptide following *in vitro* stimulation of their PBMC with HIV PR 76-84, as shown for 4 of those 6 controls (Fig. 3.1 f-i). The HIV-infected individuals tested had HLA-A2 as their only common HLA class I A or B molecule (039- A2,24; B18,73; 065- A2,3; B51,62; 078- A1,2; B18,41; 081-A2; B7,61), but to confirm that recognition of IP-30 signal sequence peptide was restricted by HLA-A2, we did cytotoxicity assays using partially matched BLCL target cells and C1R transfectants expressing either HLA-A2 or HLA-A3 as their only HLA class I molecule. Killing of target cells pulsed with the IP-30 signal sequence peptide was restricted to those target cells expressing HLA-A2 (Fig. 3.2). These data show that cross-reactive HLA-A2-restricted CTL against the IP-30 signal sequence peptide can be expanded *in vitro* by stimulating PBMC from HIV-infected individuals, but not uninfected controls, with HIV PR 76-84 peptide.

Figure 3.1. Frequency of IFN-γ-producing $CD8^+$ T cells in PBMC from HIV-infected HLA-A2 individuals in response to HIV and self-peptide. Frequency of $CD8^+$ T cells in PBMC from 4 HIV-infected HLA-A2 individuals that produced IFN-γ in response to stimulation with HIV PR 76-84, IP-30 -11 to -3 or no peptide (a) and specific cytotoxicity of T cells from those HIV-infected HLA-A2 individuals (b-e) and 4 non-HIV-infected HLA-A2 controls (f-i) stimulated *in vitro* with HIV PR 76-84 against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 -11 to -3 peptides.


HIV-infected HLA-A2+ subjects
a)
no peptide
IP-30 peptide
HIV PR 76-84
SFC / 10^6 PBMC
2000
1500
1000
500
0
039
065
078
081
HIV-infected HLA-A2+ subjects
b) 039
c) 078
d) 065
e) 081
no peptide
IP-30 peptide
HIV PR 76-84
HIV-uninfected HLA-A2+ controls
f) MG
g) SO
h) LB
i) HD
% Specific lysis
E:T ratio

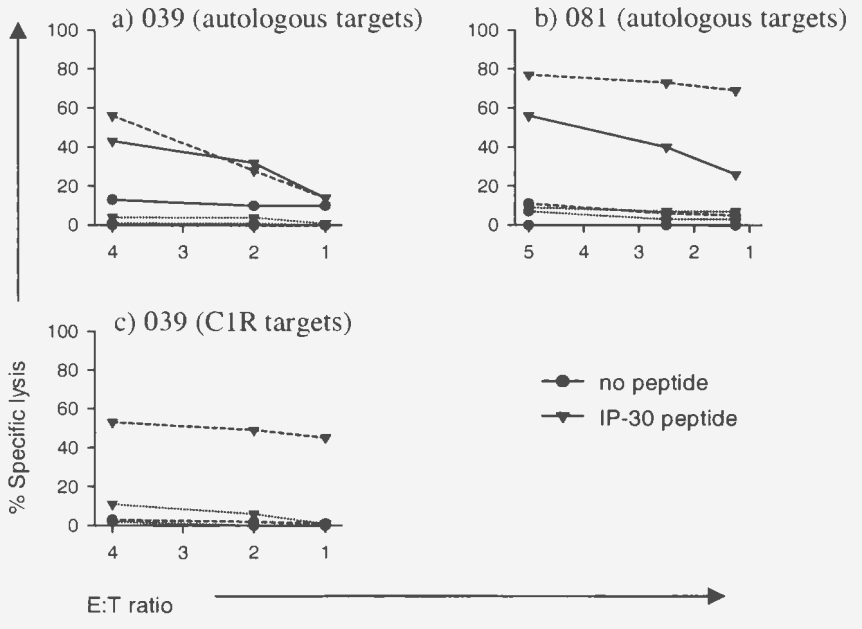


Figure 3.2. Specific cytotoxicity of T cells from HIV-infected individuals stimulated *in vitro* with HIV PR 76-84.

Specific cytotoxicity of T cells from subjects 039 (a and c) and 081 (b) stimulated *in vitro* with HIV PR 76-84 against autologous (solid lines) HLA-A2-matched (dashed lines) and mismatched BLCL (dotted lines) pulsed with IP-30 -11 to -3 peptide. Panel c shows specific cytotoxicity against peptide–pulsed C1R transfectants expressing HLA-A2 (dashed lines) or A3 (dotted lines) only.

3.4.2. IFN-γ does not indirectly activate IP-30-specific CTL

To determine whether exposure to the HIV PR 76-84 peptide could activate IP-30-specific CTL indirectly through IFN-γ production and increased IP-30 expression, we incubated PBMC with high levels of IFN-γ or with other peptides known to induce IFN-γ release *ex vivo* from the PBMC of HIV-infected individuals expressing HLA-A2. Exposure of PBMC to the same *in vitro* conditions used to activate peptide-specific CTL with addition of 500 U/mL exogenous rhIFN-γ did not induce IP-30 specific CTL (data not shown). To test whether localized endogenous IFN-γ production activated IP-30-specific CTL, we incubated PBMC from other HLA-A2 individuals with additional HIV peptides known to trigger IFN-γ production in the context of HLA-A2 or other co-expressed HLA molecules. Despite triggering *ex vivo* IFN-γ production and activating strong *in vitro* CTL responses against themselves, none of the HLA-A2-restricted reverse transcriptase (RT) 33-41 peptide (ALVEICTEM), the HLA-B44-restricted HIV RT 203-212 peptide (EELRQHLLRW) or the HLA-B57-restricted HIV Gag 147-155 peptide (LSPRTLNAW) activated CTL against IP-30 (Table 3.1). These data indicate that up-regulation of IP-30 expression through IFN-γ release does not account for *in vitro* activation of CTL against the IP-30 signal peptide with PBMC from HIV-infected individuals expressing HLA-A2.

Table 3.1. Activation of CTL by IFN-γ-inducing HIV peptides other than PR 76-84

Subject	HLA	HIV peptide	HLA restriction	SFC/10^6 PBMC[a]	Percent specific lysis[b] HIV peptide	IP-30 -11 to -3
034	A2,3; B35,60	RT 33-41	A2	330	28/15/8	7/3/0
043	A2,32; B14,62	RT 33-41	A2	65	60/44/43	5/0/3
126	A2; B44,62	RT 203-212	B44	2710	50/39/17	0/0/0
131	A2; B7,44	RT 203-212	B44	940	58/24/18	6/3/0
071	A1,2; B18,57	Gag 147-155	B57	1175	40/23/6	1/1/0
149	A1,2; B57,61	Gag 147-155	B57	325	36/20/14	0/0/1

[a]Spot forming cells were enumerated by IFN-γ ELISPOT following incubation of PBMC with the indicated peptides.

[b]Cytotoxicity following *in vitro* stimulation of PBMC with the indicated HIV peptide was measured against autologous BLCL pulsed with either the stimulating HIV peptide or IP-30 -11 to -3 signal peptide. Lysis values reported are at E:T ratios of 5:1, 2.5:1 and 1.25:1 with background lysis against unpulsed targets subtracted.

3.4.3. *In vitro* stimulation with the IP-30 self-peptide activates self-reactive and anti-HIV CTL

Although it did not trigger *ex vivo* IFN-γ release, we tested whether *in vitro* stimulation with the IP-30 signal peptide activated CTL against itself and/or the HIV PR 76-84 peptide previously shown to activate IP-30-specific CTL *in vitro*. We stimulated PBMC from the same donors who generated anti-IP-30 CTL in response to HIV PR 76-84 *in vitro* with the IP-30 signal peptide. In all 4 cases, this activated both CTL against target cells pulsed with the IP-30 signal peptide and HIV-specific CTL against HIV PR 76-84 (Fig. 3.3 a-d). At the level of *in vitro* CTL activation, there was clear cross-reactivity between HIV PR 76-84 and the IP-30 signal peptide and despite not inducing IFN-γ release *ex vivo*, the IP-30 signal peptide was effective at *in vitro* stimulation of CTL against both itself and the PR 76-84 peptide. Stimulation of PBMC from non-HIV-infected HLA-A2 controls with the IP-30 signal peptide did not activate CTL against IP-30 in any case (Fig. 3.3 e-h), indicating that *in vivo* HIV infection primes $CD8^+$ T cells for *in vitro* activation with the IP-30 signal peptide.

3.4.4. Cross-reactivity between HIV PR 76-84 and IP-30 signal peptide-specific CTL

Although the IP-30 signal peptide did not trigger *ex vivo* IFN-γ production, once PBMC from subject 081 were stimulated *in vitro* with either the IP-30 signal peptide (Fig. 3.4 a-d) or HIV PR 76-84 (Fig. 3.4 e-h), the cells did release IFN-γ when exposed to BLCL pulsed with the IP-30 signal peptide (Fig. 3.4 b and f). A similar fraction within each of the individual *in vitro* expanded $CD8^+$ T cell populations produced IFN-γ in response to either the IP-30 signal peptide or PR 76-84 peptide (Fig. 3.4 b versus c

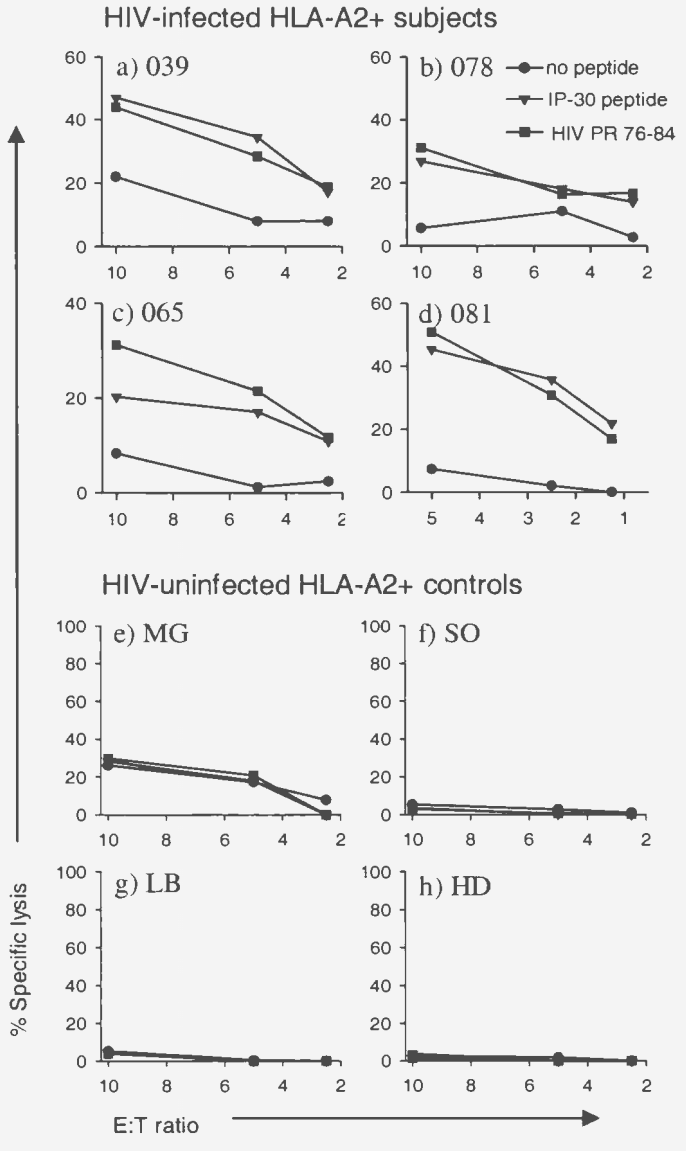
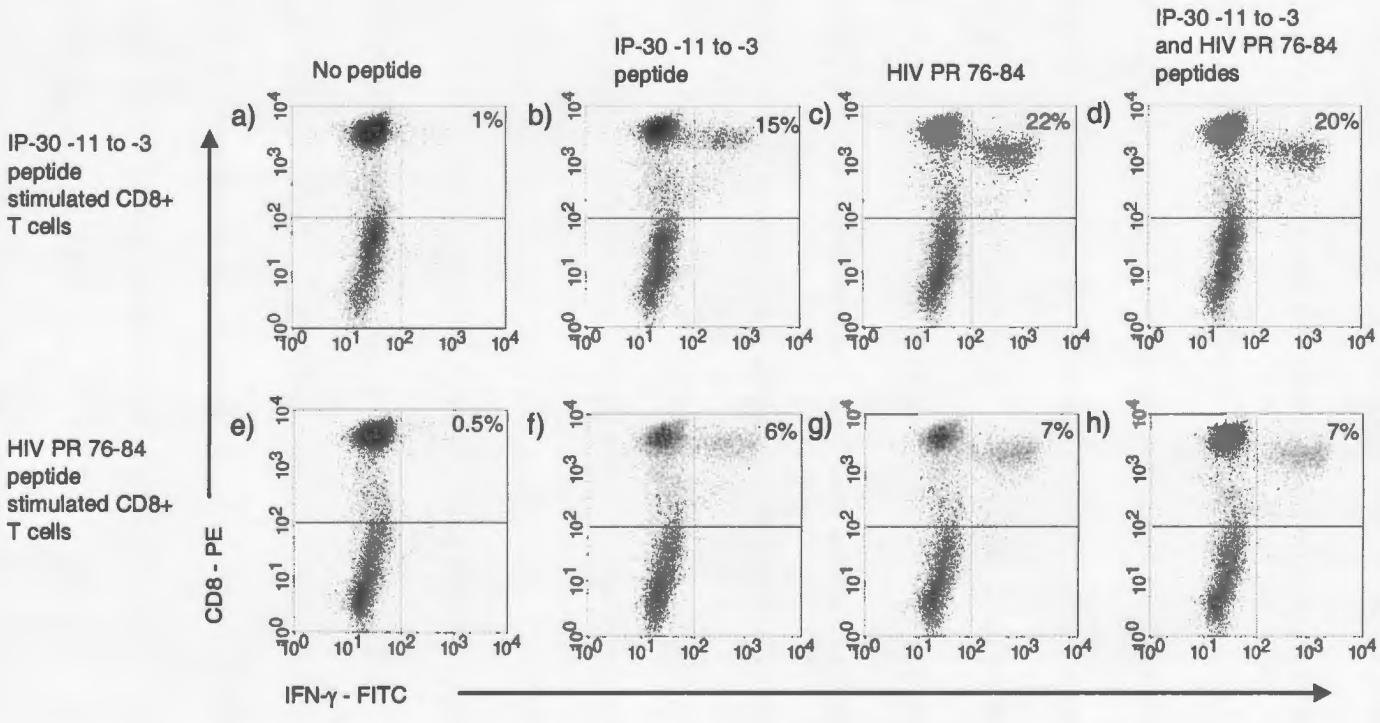


Figure 3.3. Specific cytotoxicity of T cells from HIV-infected and uninfected individuals stimulated *in vitro* with IP-30.

Specific cytotoxicity of T cells from 4 HIV-infected HLA-A2 individuals (a-d) and 4 non-HIV-infected HLA-A2 controls (e-h) stimulated *in vitro* with IP-30 -11 to -3 against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 -11 to -3 peptides.

Figure 3.4. Intracellular IFN-γ production by *in vitro* HIV PR 76-84 or IP-30 peptide stimulated $CD8^+$ T cells in response to IP-30 or HIV PR 76-84 peptides. Intracellular IFN-γ production by $CD8^+$ T cells stimulated *in vitro* for 14 days with IP-30 -11 to -3 signal peptide (a-d) or HIV PR 76-84 (e-h) and exposed for 5 hr to autologous BLCL pulsed with either no peptide (a and e), IP-30 -11 to -3 signal peptide (b and f), PR 76-84 (c and g) or pulsed with each peptide individually and combined as stimulators (d and h). The percentage of $CD8^+$ T cells producing IFN-γ is denoted in the upper right quadrant of each plot.


No peptide
IP-30 -11 to -3 peptide
HIV PR 76-84
IP-30 -11 to -3 and HIV PR 76-84 peptides
IP-30 -11 to -3 peptide stimulated CD8+ T cells
HIV PR 76-84 peptide stimulated CD8+ T cells
a) 1%
b) 15%
c) 22%
d) 20%
e) 0.5%
f) 6%
g) 7%
h) 7%
CD8 - PE
IFN-γ - FITC

and f versus g) and there was no additive effect from combining sets of stimulators pulsed separately with one or the other peptide (Fig. 3.4 d and h). The same pattern was observed with $CD8^+$ T cells from subject 039 (data not shown), suggesting a largely overlapping set of $CD8^+$ T cells responds to the different peptides.

To confirm that CTL against HIV PR 76-84 and the IP-30 signal peptide were actually cross-reactive and not reciprocally activated through some form of bystander mechanism, we did flow cytometry and depletion experiments with peptide-loaded HLA-A2:Ig dimer and carried out cold target inhibition assays. Flow cytometry with the HLA-A2:Ig dimer loaded with HIV PR 76-84 revealed a similar sized population of dimer-positive cells (~25%) following stimulation of PBMC from subject 065 with either HIV PR 76-84 (Fig. 3.5 a) or the IP-30 signal peptide (Fig. 3.5 b). The HLA-A2:Ig dimer loaded with IP-30 signal peptide failed to stain T cells within either the HIV PR 76-84 or IP-30 signal peptide *in vitro* stimulated CTL populations. Similar results were obtained with subjects 039 and 081 (data not shown). When PBMC from non-HIV-infected HLA-A2 individual MG were stimulated *in vitro* with either HIV PR 76-84 (Fig. 3.5 c) or the IP-30 signal peptide (Fig. 3.5 d), flow cytometry with the HLA-A2:Ig dimer loaded with HIV PR 76-84 revealed no dimer positive cells relative to the empty dimer control. Identical results were obtained with stimulated PBMC from non-HIV-infected HLA-A2 individual SO (data not shown).

Since the HLA-A2:Ig dimer loaded with HIV PR 76-84 stained T cells within both HIV PR 76-84 and IP-30 signal peptide stimulated CTL populations, we used this dimer for depletion experiments. Depletion of effector cells from subject 081 with the HIV

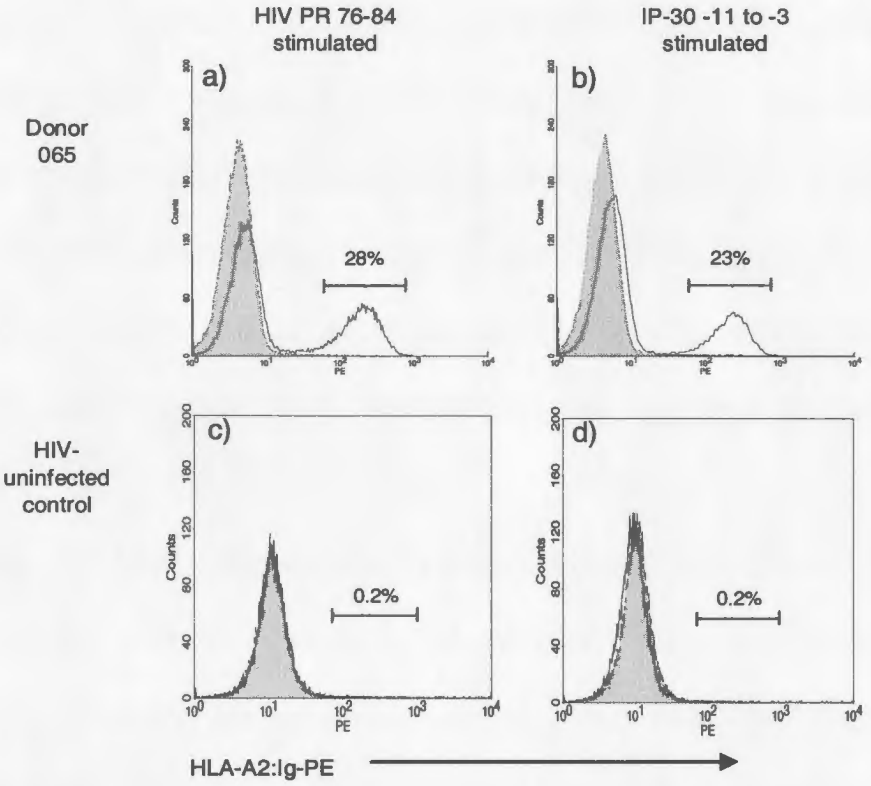


Figure 3.5. Immunofluorescent labeling of T cells from HIV-infected and uninfected individuals following *in vitro* stimulation with HIV PR 76-84 or IP-30 peptides. Immunofluorescent labeling of T cells from HIV-infected subject 065 (a and b) and non HIV-infected HLA-A2 control MG (c and d) following *in vitro* stimulation with HIV PR 76-84 (a and c) or IP-30 -11 to -3 (b and d) with HLA-A2:Ig dimer loaded with either HIV PR 76-84 (unshaded histogram) or no peptide (shaded histogram).

PR 76-84 loaded HLA-A2:Ig dimer and goat-anti-mouse IgG coupled magnetic beads reduced killing of HLA-A2 target cells pulsed with the IP-30 signal peptide to background levels. It also substantially reduced killing of HLA-A2 target cells pulsed with HIV PR 76-84, whether the effector cells were generated by stimulation with HIV PR 76-84 (Fig. 3.6a) or the IP-30 signal peptide -11 to -3 (Fig. 3.6 b). Depletion of effector cells from subjects 039, 065 and 078 produced equivalent results (data not shown).

When CTL were raised *in vitro* by stimulation with either the IP-30 signal peptide or HIV PR 76-84, killing of HLA-A2 target cells pulsed with IP-30 signal peptide or HIV PR 76-84 was effectively inhibited by cold target cells pulsed with HIV PR 76-84 (Fig. 3.6c). Cold target cells pulsed with the IP-30 signal peptide inhibited killing of HLA-A2 target cells pulsed with the IP-30 signal peptide, but had little effect on killing of HLA-A2 target cells pulsed with HIV PR 76-84 (Fig. 3.6d). Similar results were observed with CTL from subjects 078 and 081 (data not shown). Thus, both functional and phenotypic assays demonstrate cross-reactivity between the IP-30 signal peptide and HIV PR 76-84 and indicate that with PBMC from HLA-A2 HIV-infected individuals, the IP-30 signal peptide can effectively activate CTL against HIV PR 76-84 *in vitro*.

3.4.5. The CTL interaction with HLA-A2/IP-30 self-peptide is lower avidity than with HLA-A2/HIV PR 76-84

Escape of IP-30-specific CTL from thymic deletion, inability to stimulate *ex vivo* IFN-γ release, inefficient inhibition of killing with IP-30 signal peptide-pulsed cold targets and failure of the HLA-A2:Ig/IP-30 peptide dimer to stain IP-30-specific CTL

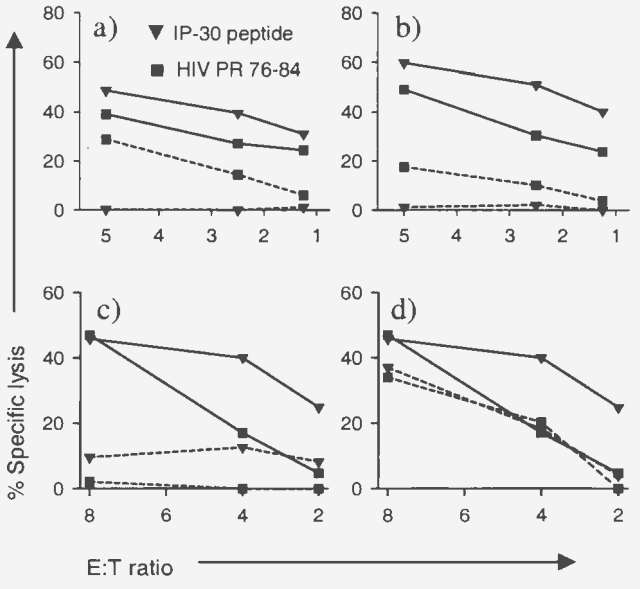


Figure 3.6. Specific cytotoxicity of effector cells following depletion with HIV PR 76-84-leaded HLA-A2:Ig dimer or cold target inhibition. Effect of depleting cells binding the HLA-A2:Ig dimer loaded with HIV PR 76-84 on specific cytotoxicity of effector cells from subject 081 stimulated *in vitro* with HIV PR 76-84 (a) or IP-30 -3 to -11 (b) against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 -3 to -11 and cold-target inhibition of cell-mediated cytotoxicity against BLCL pulsed with either the IP-30 -11 to -3 signal peptide or HIV PR 76-84. HLA-A2 expressing BLCL cold targets were pulsed with no peptide (c and d, solid lines) HIV-PR 76-84 (c, dashed lines) or IP-30 -11 to -3 (d, dashed lines). Cold targets were used at a 5:1 cold to hot target ratio to inhibit killing by HIV PR 76-84 stimulated CTL at the E:T ratios shown.

suggest that the T cell receptor interaction with the HLA-A2/IP-30 signal peptide complex is of relatively low avidity (25, 26). We compared the avidity of CTL from subjects 065 and 078 generated by *in vitro* stimulation with either HIV PR 76-84 or the IP-30 signal peptide for HLA-A2 target cells pulsed with the corresponding peptides by titrating peptide concentrations in cytotoxicity assays. Regardless of whether the CTL were generated by *in vitro* stimulation with HIV PR 76-84 (Fig. 3.7 a and c) or the IP-30 signal peptide (Fig. 3.7 b and d), killing of target cells pulsed with HIV-PR 76-84 fell to 50% of baseline killing at < 0.2 nM HIV-PR 76-84, an approximately 1000-fold lower peptide concentration than did killing of target cells pulsed with the IP-30 signal peptide. While a lower affinity of HLA-A2 binding for the IP-30 peptide than HIV PR 76-84 could also explain these results, this is unlikely for several reasons. Firstly, IP-30 was identified as a very prominent self-peptide in the context of HLA-A2 molecules purified from lymphoid cells (18). Secondly, the IP-30 signal peptide sequence fits the motif for HLA-A2 binding and ranks higher than HIV PR 76-84 using the 2 most common algorithms for predicting HLA binding affinity. With http://bimas.dcrt.nih.gov/molbio/hla_bind site, IP-30 -11 to-3 scores 47.3 and HIV PR 76-84 scores 1.6. Using the Rammensee lab developed site http://www.syfpeithi.de, the IP-30 -11 to-3 and HIV PR 76-84 peptides score 28 and 18 respectively.

3.5. Discussion

The potential for molecular mimicry to activate cross-reactive T cells during viral infections relates to the extent of peptide mimicry, from the perspective of host HLA molecules and T cell receptors, and to the degree of self-tolerance imposed upon T cells

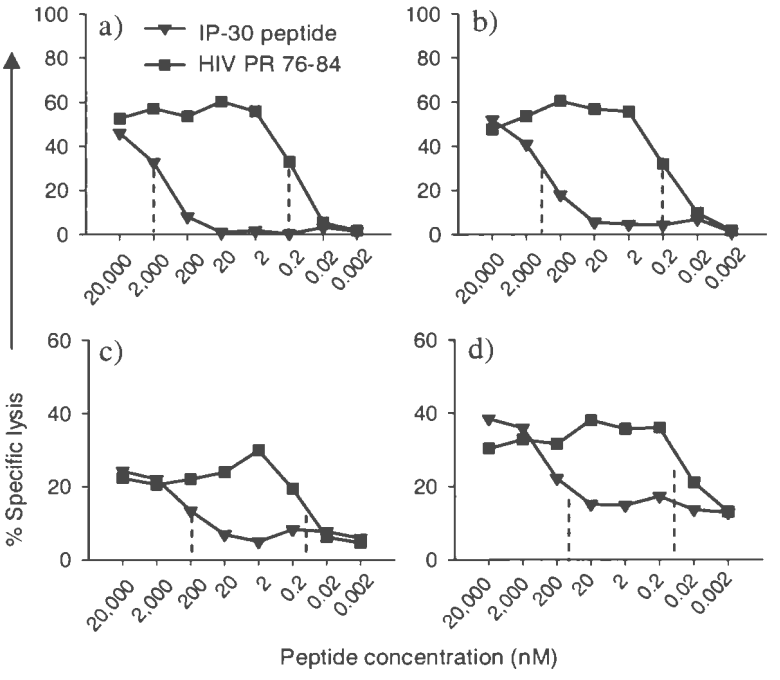


Figure 3.7. Peptide titration curve for cytotoxicity of effector cells activated by *in vitro* stimulation with HIV PR 76-84 or IP-30 peptides. Peptide titration curves for cytotoxicity of effector cells from subject 078 (a and b) and 065 (c and d) activated by *in vitro* stimulation with HIV PR 76-84 (a and c) or IP-30 -11 to -3 (b and d) at an E:T ratio of 5:1 against autologous BLCL targets pulsed with decreasing concentrations of either HIV PR 76-84 or IP-30 -11 to -3 as indicated by the legend. Dashed vertical lines to the X axis indicate the peptide concentrations at which killing of peptide targets fell to 50% of maximum values.

reactive with the analogous self peptides. Self-reactive T cells that avoid thymic and peripheral deletion may persist in the periphery in an effectively tolerant state invoked by their inactivation, suppression or ignorance. While this is normally a stable situation, perturbations accompanying viral infections, including the introduction of more immunogenic variants of self peptides, may challenge the tolerance imposed on self-reactive T cells. In this study, we found that following HIV infection, $CD8^+$ T cells reactive against a self peptide derived from IP-30 could be readily activated and expanded *in vitro* by exposure to either the foreign HIV PR 76-84 peptide or to the self IP-30 -11 to -3 signal peptide. Despite acquiring the capacity to expand and differentiate into CTL *in vitro* in response to self peptide, the $CD8^+$ T cells cross-reactive with HIV PR 76-84 didn't release IFN-γ *ex vivo* when exposed to the self peptide. This presumably reflects the relatively low avidity of the TCR/HLA-A2/IP-30 signal peptide interaction that allowed the IP-30-specific T cells to originally escape thymic deletion and persist in the periphery without effecting symptomatic autoimmunity (25,26). Low avidity relative to the HIV PR 76-84 peptide recognition was reflected in an approximately 1000-fold lower concentration of HIV PR 76-84 than IP-30 -11 to -3 required to trigger 50% maximal lysis of peptide-pulsed targets, inefficient inhibition of cytotoxicity against targets pulsed with HIV PR 76-84 by cold targets pulsed with IP-30 -11 to -3 and failure of the HLA-A2:Ig dimer loaded with IP-30 -11 to -3 to stably bind the cross-reactive $CD8^+$ T cells.

The higher avidity interaction with HIV PR 76-84 expands cross-reactive $CD8^+$ T cells specific for the IP-30 signal peptide *in vivo*, and in so doing, increases their sensitivity to activation and expansion by the IP-30 signal peptide itself, at least *in vitro*.

This situation, effected by viral infection, parallels a proposed approach to therapeutic vaccination for cancer where synthetic peptide variants are used to activate T cells against self-peptides expressed on tumor cells, when the self-peptides themselves do not activate the T cells (27). In these cases, self-reactive T cells react with higher avidity against synthetic heteroclitic variant peptides and once activated and expanded by exposure to the variants, selectively attack tumor cells, which express higher levels of the analogous self peptide than do normal host cells (27). In this study, we identified a viral peptide that acts as the synthetic heteroclitic variant activating self-reactive T cells against a self-peptide expressed at higher levels in lymphoid cells exposed to IFN-γ. Activation, by HIV PR 76-84, of cross-reactive CTL against an IFN-γ-inducible protein widely expressed in lymphoid cells could have dangerous implications for autoimmunity, especially when elevated IFN-γ levels during periods of viral replication upregulate IP-30 expression (17). As in the selective targeting of tumor cells by autoreactive CTL, activation and proliferation of IP-30-specific CTL against a backdrop of elevated IFN-γ levels could selectively target IFN-γ exposed APC expressing elevated levels of IP-30 and thereby contribute to the APC dysfunction and lymphoid architectural destruction characteristic of progressive HIV infection. However, there is as yet no evidence of any relationship between HIV disease progression and CTL against HIV PR 76-84. Of 46 HLA-A2 HIV-infected individuals we tested by IFN-γ ELISPOT, 17 had T cells reactive with HIV PR 76-84 and 7 of these 17 generated HIV PR 76-84 and IP-30 -11 to -3 cross-reactive CTL following *in vitro* peptide specific stimulation. While it is clearly a subset of HLA-A2 HIV-infected individuals that possess these CTL, there was no clear

indication that this subset had a clinically distinct pattern of disease progression and longitudinal studies will be needed to address this issue.

Current models of T cell selection hold that $CD8^+$ thymocytes are positively selected through interaction with self-peptides ubiquitously expressed with the MHC class I molecules of cortical epithelial cells. Subsequent negative selection eliminates only those $CD8^+$ T cells that react with high avidity against endogenous peptide/MHC class I molecule complexes expressed on bone marrow-derived APC at the cortico-medullary junction or in the medulla (25). Therefore, selection of $CD8^+$ T cells with the capacity for high avidity interactions with foreign peptide/MHC class I complexes is absolutely dependent upon those $CD8^+$ T cells engendering low avidity self-reactivity. Within the normal environmental milieu, such low avidity self-reactive T cells are non-pathogenic, however, increased expression of the self-peptide, alterations in the environment or activation, proliferation and differentiation of self-reactive CTL in response to foreign peptides could shift the situation towards symptomatic autoimmunity. The ability of the IP-30 signal peptide itself to activate and expand CTL *in vitro*, once they have been activated *in vivo* by HIV PR 76-84, suggests that the *in vivo* activation and or expansion of cross-reactive CTL lowers the threshold for breaking the effective tolerance of these T cells for self tissue. While the potential negative consequence of CTL against uninfected lymphoid cells is obvious, as long as the relatively low avidity of CTL against IP-30 protects against symptomatic autoimmunity, there could be substantial advantages to cross-reactivity between anti-viral CTL and a prominent self peptide up-regulated by pro-immune cytokines such as IFN-γ. One previously identified positive

consequence of activation of low avidity self-reactive T cells can be selective targeting of tumor cells. In the case of the self-peptide we identified as functionally analogous to an HIV CTL epitope, its broad distribution in lymphoid cells, prominent basal representation in HLA-A2 molecules and up-regulation in response to IFN-γ could all potentially benefit the anti-HIV CTL response. Based on the premise that cross-reactive self-peptides are required for homeostatic maintenance of anti-HIV memory T cells when HIV replication is undetectable, the broad distribution and prominent representation of IP-30 -11 to -3 may convey an advantage on anti-HIV CTL manifesting lower avidity recognition of that particular self peptide. In this context, we observed a persistent high frequency of $CD8^+$ T cells against HIV PR 76-84 in one individual (subject 081), despite antiretroviral suppression of HIV replication to undetectable levels for over 6 years. When HIV replication or any other event that elevates IFN-γ levels occurs, the selective up-regulation of IP-30 might favor propagation of those anti-viral T cells with the capacity for functional recognition of IP-30 -11 to -3. The differential avidity would ensure an impact only on those cross-reactive memory T cells previously activated by higher avidity interactions with foreign peptides and possibly allow a self peptide to selectively activate anti-viral CTL. Thus, both in terms of their homeostatic maintenance and the potency of their response to secondary challenge, CTL reactive with self peptides such as IP-30 -11 to -3 may have a significant advantage.

Although we identified CTL against IP-30 -11 to -3 specifically through their cross-reactivity with HIV PR 76-84, it is quite feasible that a variety of CTL restricted to HLA-A2 would have lower avidity interactions with IP-30 -11 to -3. Minimal sequence

homology between IP-30 -11 to -3 and HIV PR 76-84 as well as between other reported cross-reactive CTL epitopes such as the influenza A matrix protein peptide MI 58-66 (GILGFVFTL) and HIV p17 77-85 (SLYNTVATL) illustrate the surprising level of promiscuity of $CD8^+$ T cell recognition of peptides with a common restricting element (28). Previous studies showed that a broad T cell receptor repertoire is selected even when thymic presentation is restricted to a single MHC/peptide combination (29). The surrogate function of self-peptides in positive selection of T cells with high avidity for foreign peptides may be recapitulated in the periphery following initial activation of these T cells during infection if the expression pattern of the surrogate self peptide is appropriate. Depending upon the relative stringency of requirements for expression levels and avidity threshold to serve as a positively selecting surrogate peptide in the periphery versus during thymic selection, the autoreactivity that drives positive selection of the T cell repertoire may also play a major role in evolution of the T cell response against foreign agents.

In summary, this study demonstrates cross-reactivity between HIV PR 76-84 and IP-30 -11 to -3 at the level of $CD8^+$ T cell recognition. The interaction with IP-30 is lower avidity and the $CD8^+$ T cells require *in vivo* priming with HIV PR 76-84 in order to respond to IP-30 -11 to -3 *in vitro*. Although such cross-reactivity raises the possibility of autoreactivity against APC, in the context of our current understanding of T cell selection, the IP-30 -11 to -3 signal peptide appears to be an ideal surrogate peptide for selection, maintenance and expansion of $CD8^+$ T cells against foreign agents.

3.6. Acknowledgements

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4. Thesis Summary

4.1. Summary

This was a proof-of-concept study to determine whether mutations conferring resistance to commonly prescribed antiretroviral drugs could sustain or enhance immune recognition of HIV. As described in chapter two, we tested wild-type and variant HIV CTL epitopes incorporating drug resistance mutations and identified several mutations which were able to sustain CTL recognition in HIV-infected donors. The variant-specific responses we detected represent novel HIV CTL epitopes which are recognized in the context of common alleles HLA-A2, -A3, -B35 and -B44. We detected CTL responses to epitopes containing single as well as dual amino acid substitutions conferring resistance to antiretroviral drugs. A significant fraction of donors responded to these variant CTL epitopes and, in most cases, recognition of specific drug resistance mutations was associated with exposure to the appropriate drug that selected the mutation. In some instances, CTL epitopes containing drug resistance mutations were more immunogenic than corresponding wild-type consensus epitopes. Thus, we demonstrated that HIV drug resistant variants which emerge under the selective pressure of antiretroviral therapy can sustain and, in some cases, enhance recognition by CTL.

Demonstrating immunogenicity of drug resistant HIV may have important implications for improving the long-term efficacy of antiretroviral therapy. Treatment of HIV-infected individuals often involves sequential and alternating combinations of NRTI, NNRTI and PI, which can result in high level resistance to drugs within all three classes of inhibitors. Resistance to novel anti-HIV compounds has also been documented. The fusion inhibitor Enfuvirtide which was recently introduced into clinical practice and the

integrase inhibitor S-1360 presently in clinical trials have both been shown to select for resistance mutations (1, 2). Identification of drug resistance mutations that can sustain or enhance CTL recognition provides evidence in support of developing treatment and vaccine strategies which combine immune and drug selection pressures to prevent emergence and transmission of drug resistant HIV. Ultimately, this could delay or even prevent the outgrowth of drug resistant variants, which is the primary cause of treatment failure in HIV-infected individuals.

We also detected strong CTL responses against an HIV-unrelated control peptide in several HIV-infected donors. This peptide was derived from IP-30, a self protein involved in antigen processing and presentation (3). Since IP-30 peptide-specific CTL were only detected in HIV-infected donors, we investigated the basis for activation of these self-reactive CTL. Chapter 3 outlines our results showing that functional cross-reactivity between an HIV protease peptide and the IP-30 self-peptide led to the activation of these self-reactive CTL. Cross-reactivity between an HIV peptide and a prominent self-peptide indicates the potential for HIV-specific CTL to contribute to prominent immunopathology in HIV infection. However, although IP-30-specific CTL were generated at high frequencies in HIV-infected donors, they did not produce any apparent immunopathology. Conversely, cross-reactivity of HIV peptide-specific CTL for a self-peptide may allow for continuous antigenic stimulation to maintain anti-HIV memory CTL when HIV replication is suppressed by ART.

The results presented in the second chapter might be important for the development of novel anti-HIV treatment and vaccine strategies which combine immune and drug selection pressures. For individuals on ART, emergence and accumulation of

drug resistance mutations is a significant obstacle to long-term suppression of HIV replication. As well, since $CD4^+$ T cell help is critical for maintaining the effectiveness of HIV-specific CTL (4), progressive loss of $CD4^+$ T cells may impair generation of CTL responses against novel epitopes generated by viral mutations as disease progresses. Early treatment with drugs that select for resistance mutations that sustain or enhance recognition by HIV-specific CTL may allow targeting of drug resistant HIV before loss of $CD4^+$ T cells can impair CTL function. Furthermore, wild-type and corresponding variant epitopes incorporating drug resistance mutations that sustain or enhance recognition by CTL may be incorporated into a HIV vaccine to generate pre-existing immunity against wild-type and drug resistant HIV. In the event of failure to protect against HIV infection, subsequent treatment with drugs that select for resistance mutations susceptible to CTL may allow for long-term immune control of HIV, especially if those CTL are pre-activated by immunization.

While we have laid the basis for recognition of CTL epitopes incorporating drug resistance mutations which suggests potential efficacy of convergent immune and drug-based treatment and vaccine strategies, several important questions remain. First, is there a correlation between CTL recognition of drug resistant HIV and improved immune containment of HIV in treated individuals? Due to the limited number of epitopes examined and small sample size of our cohort, we were unable to provide statistically significant evidence for this. However, broad HIV-specific CTL responses which cross-react with potential escape mutations are thought to contribute to prolonged immune control in the absence of antiretroviral therapy (5). Therefore, we anticipate improved control of HIV replication in treated individuals who exhibit CTL recognition of epitopes

incorporating drug resistance mutations. Furthermore, there may also be selection against drug resistance mutations when they are more immunogenic than corresponding wild-type epitopes, thus rendering HIV more susceptible to antiretroviral drugs.

Second, is there evidence for viral mutations that simultaneously escape both immune and drug selection pressures? HIV has an extraordinary capacity to mutate and prolonged viral exposure to both immune and drug selection pressures may result in further mutations providing complete escape from both. The functional importance of HIV *pol* gene products, as well as selection and maintenance of drug resistance mutations which impair viral fitness implies that there are strict limitations on sequence diversity of the *pol* gene. This suggests that combining immune and drug selection pressures may provide sufficient constraints to prevent or delay escape from dual immune and drug selection pressures.

Third, we focused our study on epitopes incorporating drug resistance mutations which were able to sustain or enhance CTL recognition. Mutations within CTL epitopes can also generate altered peptide ligands which may lead to TCR antagonism of CTL (6). Altered peptide ligands can bind to and drive the proliferation of wild-type specific CTL which may fail to kill target cells presenting the altered peptide, resulting in failure to elicit variant peptide-specific CTL (7). Altered peptide ligands may also serve as decoy peptides which retain the ability to interact with wild-type specific CTL and prevent targeting of corresponding wild-type epitope sequences (8, 9). Further studies on the interaction of CTL epitopes incorporating drug resistance mutations with wild-type-specific CTL should establish whether these mutations can generate altered peptide ligands that antagonize the CTL response to either wild-type or drug resistant HIV.

Lastly, the results of this study were based on CTL responses in a cohort of HIV-1 clade B infected individuals. Most HIV infections that account for the global epidemic are comprised of non-clade B infections which occur primarily in Africa and Asia. Compared with donors in our cohort, populations within these regions are exposed to different HIV-1 clade isolates and show some differences in HLA allele frequencies. Therefore, it will be important to test CTL recognition of drug resistant variants in different ethnic populations in the context of HLA alleles and HIV-1 clade isolates that predominate in these populations.

4.2. Future directions

The most prevalent HIV-1 clade circulating in North America and Europe (clade B) represents just one of multiple clades circulating worldwide. Within subgroup M, the major group of HIV-1, eleven distinct clades (A1, A2, B, C, D, F1, F2, G, H, J and K) occur with varied frequency in different geographic regions. Recent estimates indicate that less than 15% of worldwide infections are due to HIV clade B, while nearly 75% of new HIV infections are due to subtypes A and C (10). Circulating recombinant forms (CRF) of HIV generated by recombination of distinct subtypes as well as viral quasispecies generated following emergence and accumulation of viral mutations within HIV-infected individuals also contribute to the global diversity of HIV. This enormous variability of HIV poses a significant challenge for the development of a globally effective HIV vaccine or any broad-based application of vaccines targeting drug resistance mutations.

Broad cross-reactivity of HIV-specific CTL epitopes has been observed in clade B and non-clade B infected individuals (11, 12) and may account for the improved clinical

outcome of some HLA-B57^{+} long term nonprogressors (LTNP) (5). However, cross-clade recognition of HIV-specific CTL epitopes incorporating drug resistance mutations has not yet been studied. Comparison of commonly circulating clades shows that some CTL epitopes share complete sequence homology, while for others there is only partial sequence homology. We have already demonstrated that HIV-1 clade B CTL epitopes incorporating drug resistance mutations sustain antigenicity in HIV-infected individuals. Since several mutations conferring resistance to antiretroviral drugs are conserved across multiple clades (13), we sought to determine whether CTL activated in HIV-1 clade B infected individuals cross-react with corresponding clade A and C wild-type and variant peptides incorporating drug resistance mutations.

Based upon the epitopes to which we observed strong CTL responses in our cohort of HIV-1 clade B infected individuals, we tested recognition of corresponding epitopes from clades A and C, two of the most prevalent HIV-1 clades circulating in Africa (R. Mason, unpublished data). Comparison of the consensus clade B CTL epitopes PR 76-84, RT 33-41, RT 107-115, RT 179-187 and RT 203-212 CTL with consensus clade A and C sequences showed complete sequence homology across all three clades for PR 76-84 and RT 107-115. However, only partial homology across clades A, B and C was observed for RT 33-41, RT 179-187 and RT 203-212 which are restricted by HLA-A2/-A3, -A2 and -B44, respectively. Since these HLA types also occur in the majority of individuals where HIV clades A, B and C predominate, these epitopes were chosen for analysis of cross-clade recognition of HIV-1 CTL epitopes incorporating drug resistance mutations.

A significant fraction of donors had HIV-1 clade B-specific CTL that cross-reacted with corresponding clade A and C wild-type and variant peptides. For the HLA-A2/A3-restricted RT 33-41 epitope, several donors with clade B RT 33-41-specific CTL also recognized at least one clade A or C wild-type or M41L variant peptide. Among HLA-$A2^+$ donors RT 33-41 clade B peptide-specific CTL were primarily cross-reactive for clade A derived RT 33-41 wild-type and M41L variant peptides with limited cross-reactivity for clade C derived wild-type and M41L variant peptides. In the context of HLA-A3, CTL were fully cross-reactive for clade A and C wild-type and M41L variant peptides. Thus, for the RT 33-41 sequence, HLA-A3-restricted CTL displayed broader cross-clade reactivity for corresponding wild-type and variant peptides from clades A and C compared with HLA-A2-restricted CTL.

Although cross-clade recognition of RT 179-187 was detected in only one donor, we later observed that this epitope, which has been documented to be an HLA-A2-restricted epitope, was also recognized by several HLA-$A3^+$ donors. Therefore, further testing of clade A and C wild-type and variant RT 179-187 peptides may reveal broader cross-clade reactivity in the context of HLA-A3.

Finally, a number of donors with clade B RT 203-212 wild-type or variant-specific CTL also recognized corresponding wild-type clade A or C derived peptides. For some donors, the corresponding clade C-derived wild-type peptide was more immunogenic than the homologous clade B epitope. Surprisingly, we also observed HLA-$B44^+$ donors who responded to this wild-type clade C epitope although they did not recognize either the wild-type or variant epitope sequence from clade B.

Viral genotyping confirmed clade B infection for all donors in our cohort. Thus, our preliminary results indicate that cross-clade CTL recognition of wild-type and variant peptides incorporating drug resistance mutations can occur even in the absence of exposure to multiple HIV clade variants. Furthermore, the observation that RT 33-41 and RT 179-187 were able to elicit strong CTL responses in the context of both HLA-A2 and -A3 renders them ideal targets for treatment and vaccine strategies aimed at eliciting HIV-specific CTL since both alleles are common within different ethnic populations. Finally, it will also be important to test additional HIV-specific CTL epitopes overlapping common drug resistance mutations restricted by HLA alleles that occur commonly in regions where non-clade B HIV isolates predominate.

With over ten major HIV-1 clades distributed at varying frequencies in different geographic regions, protection against multiple clades will be essential for any broadly applicable HIV treatment or vaccine strategy. The increasing prevalence of CRFs of HIV-1 as well as documented cases of co-infection and superinfection with distinct clades demonstrates that exposure to multiple clades is not uncommon (14-17). An effective immune response directed against one HIV-1 clade or variant does not necessarily protect against subsequent infection with a different clade or even a closely related strain (18). Epitopes which elicit broad cross-clade CTL responses are more likely to afford protection against multiple clades.

Our preliminary findings demonstrate the occurrence of cross-clade CTL recognition of HIV epitopes overlapping sites of drug resistance mutations which can be used to address the problem of HIV-1 clade diversity. Anti-HIV-specific CTL directed against multiple conserved epitopes have been documented in LTNP and may contribute

to their slow disease progression (5). Thus, it may be possible to provide antiretroviral therapy for HIV-infected individuals which selects for drug resistance mutations that sustain CTL recognition of wild-type and variant epitopes from multiple clades, in order to protect against heterologous HIV-1 clades and prolong the effectiveness of antiretroviral treatment. CTL epitopes incorporating drug resistance mutations that elicit broad cross-clade reactivity for wild-type and drug resistant variants from multiple clades are also ideal for inclusion in CTL-inducing vaccines to prevent selection and transmission of heterologous HIV-1 clade isolates.

4.3. Conclusions

We identified six common antiretroviral drug resistance mutations that sustained and in some cases, enhanced the antigenicity and immunogenicity of HIV-1 *pol* CTL epitopes. Variable patterns of cross-reactive and selective recognition of wild-type and corresponding variant epitopes indicated a relatively diverse population of $CD8^+$ T cells reactive against these epitopes. The frequency and diversity of CTL reactivity against the variant peptides incorporating drug resistance mutations and their ability to activate and expand CTL precursors *in vitro* illustrates a significant functional interface between the immune system and antiretroviral therapy. Thus, drug resistant variants of HIV are susceptible to immune selective pressure which could be applied to combat transmission or emergence of antiretroviral drug resistance and to enhance the immune response against HIV.

Stimulation of PBMC from HLA-A2 HIV-1-infected individuals with the HLA-A2-restricted HIV PR peptide 76-84 activated CTL against the IP-30 signal self-peptide. The IP-30 signal peptide also activated CTL *in vitro* against itself and HIV PR 76-84

suggesting T cell cross-reactivity between the two peptides. As well, the T cell interaction with HIV PR 76-84 was higher avidity than with the IP-30 signal peptide, indicating that HIV PR76-84 is a heteroclitic peptide variant of the IP-30 signal peptide, which has implications for immune memory and autoimmunity.

4.4 Significance of Research

HIV treatment and vaccine strategies that combine immune and drug selection pressures could become increasingly important as the epidemic progresses due to the rapid evolution of HIV. Transmission and replication of HIV within the population has resulted in selection pressure on the virus to escape HLA-mediated immune responses (19, 20). The most frequent HLA alleles within infected populations are more likely to have influenced viral adaptation through mutations in CTL epitopes restricted by common HLA alleles. Therefore, it may be beneficial to use antiretroviral drugs to select for mutations that enhance CTL recognition in individuals with common HLA alleles. Further analysis of the effect of drug resistance mutations on CTL recognition throughout the entire *pol* gene may reveal novel epitopes incorporating drug resistance mutations which lie outside of previously defined consensus epitopes.

Since the first reported case of HIV over twenty year ago, more than 20 million people have died from HIV/AIDS. The Joint United Nations Programme on HIV/AIDS estimated over 3 million AIDS related deaths and nearly 5 million new HIV infections in 2004, indicating that the best hope for bringing an end to this epidemic is a vaccine that can provide sterilizing immunity against HIV. Unfortunately, there are numerous obstacles facing the development of an HIV vaccine. To date, only two candidate HIV vaccines have advanced through phase III clinical trials in humans and both ultimately

failed to demonstrate protection against HIV (21, 22). In addition to safety and efficacy, other issues such as affordability, ease of administration and distribution to populations most severely affected by HIV/AIDS will likely take many years to resolve. In the meantime, there is an urgent need to treat the nearly 40 million people currently infected with HIV and the many more who are likely to become infected before a successful prophylactic vaccine becomes available. Our results may contribute to the development of novel HIV treatment and vaccine strategies that may facilitate more effective management of HIV infection and, therefore, towards the long-term survival of HIV-infected individuals.

4.5. References for Summary

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