HIV: A fugitive from cellular immune responses

More than 40 million people are infected with HIV worldwide. In almost all cases, untreated HIV infection leads to profound immunodeficiency and death. Despite the uniformity in clinical endpoint, HIV tailors itself to each individual host through unrelenting, error-prone replication(1). The specific adaptation of HIV to its host enables the virus to rapidly acquire drug resistance, alter its target cell tropism, and specifically evade antiviral immune responses(2).

Cellular immune responses are particularly sensitive to HIV evolution. In both HIV(3) and simian immunodeficiency virus (SIV) infections(4), the majority of viral changes that accrue outside of the viral envelope during natural infection confer resistance to cytotoxic T lymphocytes (CTL). Escape mutants can arise in any viral protein and develop throughout infection, with the first mutants arising only days after CTL responses emerge(5, 6).

Rapid CTL escape correlates with high CTL efficacy

As candidate HIV vaccines increasingly focus on eliciting CTL responses(7), there is an urgent need to identify CTL with the best antiviral potential. The overall CTL response to HIV is complex, consisting of T cells recognizing epitopes with varying degrees of success(8). There is tantalizing preliminary evidence that CTL responses which select escape variants rapidly have superior antiviral efficacy. In a comparison of three CTL responses, CTL raised against an epitope in Nef more effectively reduced viral replication than CTL responses against epitopes in either Gag or Pol (8). Escape variants arose in the Nef-specific CTL very rapidly(9) whereas only sporadic variation was observed in the Gag epitope in tissue culture(9). Though the in vivo kinetics of escape within the Nef epitope are not known, the Gag response with modest antiviral effect selects for escape variants very slowly in HIV+ people(10).

In comparable studies of SIV-infected monkeys, the CTL specificity with the greatest antiviral suppressive capacity(11, 12) selects escape variants during acute infection(5). Taken together, these studies suggest that selection of escape variants during early HIV infection is a molecular signature of an effective CTL response.

The Challenge of Studying Early Events in HIV Infection

The biology of HIV complicates studies of early infection. Flu-like symptoms and malaise(13) are the most common clinical indicators of primary HIV infection, but these signs are non-specific and occur only in a subset of individuals. Most newly infected individuals, therefore, are unaware of their acute infections and are rarely diagnosed. Indeed, up to 80% of HIV+ men do not know they are infected(14). By the time most HIV+ individuals seek physician care, the duration of their HIV infection is unknown. Consequently, most HIV research utilizes samples from chronic infection, collected after patients learn of their HIV status and receive medical care. Unfortunately, this makes it very difficult to study effective CTL responses that select escape variants during early infection.

The latent reservoir as a window into early HIV infection

Soon after the introduction of highly effective drug therapies for HIV, scientists discovered that a "reservoir" of memory CD4+ T cells infected with HIV persists indefinitely even when HIV virions cannot be detected in blood plasma(15). The reservoir is seeded throughout infection and is extremely long-lived(15) with a half-life of approximately 44 months(16). Complete eradication of virus with drugs alone is unlikely because of the longevity of the latent reservoir(16, 17).

In light of the reservoir's importance for antiretroviral treatment strategies, several groups have examined the sequence composition of viruses in the latent reservoir. Unlike the short-lived HIV virions found in blood plasma, the reservoir is comprised of quiescent cells infected at different stages of infection. The complex and variable population of sequences in latently infected cells thus represent a living archive of HIV's evolution within an individual. Within this archive, viruses from acute infection may be overrepresented because the viral load during this period is 100-1000-fold higher than the viral load during chronic infection. In support of this idea, drug-
susceptible virus sequences can be found in the latent reservoir of drug-resistant patients with complex treatment histories (Figure 1)(18).

We reason that the emergence of drug resistance is fundamentally similar to the emergence of CTL escape, and that the latent reservoir also sequesters viruses prior to the emergence of CTL escape variants. If this is true, mining this reservoir will enable identification of escape variants that emerge during early infection. This approach may allow for the comprehensive identification of CTL responses that exert strong selective pressure on HIV during early infection using widely available samples from chronically infected individuals.

Specific Aim 1: Defining the optimal conditions for recovering virus from the latent reservoir

Rationale for optimizing latent reservoir extraction strategies in SIV+ macaques

Though latently infected cells are present in all HIV+ individuals(19), their frequency is very low. Therefore, selectively purifying these cells is a considerable technical challenge. To do this, we will prototype latent reservoir extraction strategies using samples from simian immunodeficiency virus (SIV) infected rhesus macaques. Samples from nearly 200 animals infected with the genetically uniform, molecularly cloned SIV strain SIVmac239 (20) are available for these studies. SIVmac239-infected macaques are particularly attractive for CTL escape studies(5, 21) because of the sequence homogeneity of the infecting virus, the large number of known CTL responses with defined MHC restriction(22, 23), and the availability of methods of rapidly sequencing the entire SIVmac239 genome(4).
Figure 2 shows the pattern of CTL escape in the plasma virus of a single SIVmac239 Tat CTL epitope (Tat SL8). Immediately after infection, the predominant sequence in the animal's virus matches the SIVmac239 inoculum. The objective of our latent reservoir enrichment is to maximize recovery of viruses that possess this wild-type SIVmac239 sequence from samples collected more than one year after SIV infection. If this is successful, we will use the same strategy to purify latent reservoir-containing cells from HIV-infected persons.

The kinetics of escape within the Tat CTL epitope will be important if we are unable to recover latently infected cells possessing wild-type SIV sequences. We can sequence SIV in the plasma at multiple timepoints throughout infection and use these sequences to estimate when cells in the latent reservoir became infected. Using the example shown in Figure 2, viruses entering the reservoir between 8 and 54 weeks post-infection are expected to possess a proline for serine substitution at position 1 of the epitope (p1:S->P). If the P1 substitution is coupled with a p4:E->G substitution, we can infer that the cell containing this virus entered the reservoir between 64 and 88 weeks post-infection.

**Strategy for optimization in rhesus macaques**

Our initial strategy for sequencing virus from latently infected cells will be based on a virus production assay devised by Monie and colleagues(19)(Figure 3). We will first obtain resting, memory T cells using magnetic cell sorting (Miltenyi). We will purify CD4+ T cells from peripheral blood mononuclear cells and remove CD45RA+/CD62L+ naïve and CD25+/CD69+/HLA-DR+ activated T cells. If this does not produce a population of cells with sufficient purity as assessed by flow cytometry, we can also acquire our population of interest with a Cytomation MoFlo flow cytometer/sorter.

We will differentiate the integrated proviruses containing the archival virus of interest from preintegrated viruses that are often found in newly infected CD4+ T cells and thus have sequences similar to the circulating plasma virus. Addition of antiretroviral drugs (3TC, Tenofovir, Efavirenz, and an integrase inhibitor) to the culture media inhibits the reproduction of these newly infected preintegrated viruses. To stimulate the cells to produce the integrated provirus while in the presence of the antiretrovirals, we will co-culture the purified resting, memory CD4+ cells with irradiated non-infected PBMC, phytohemagglutinin, and IL-2. Tissue culture supernatant will be obtained at three day intervals for two weeks and viral RNA will be isolated. The first exon of tat will be RT-PCR amplified and cloned from viral RNA. At least 24 clones per timepoint will be analyzed by sequencing. If the assay is performed successfully, we expect to observe a complex population of sequences within the Tat SL8 CTL epitope including sequences identical to plasma virus sequences from earlier timepoints during infection.

**Specific Aim 2: Reconstruction of archival virus in the latent reservoir of HIV+ individuals**

We are currently collecting samples from HIV+ volunteers to study CTL epitope sequences within the latent reservoir. This project is being undertaken in collaboration with Dr. James Sosman, an HIV specialist at UW-Madison Hospitals and Clinics, and research nurse Jennifer Bellehumeur. To our knowledge, this is the only translational HIV/AIDS research collaboration at UW-Madison.

Since we expect the pattern of CTL escape to vary from patient to patient, we will focus our initial efforts on characterization of variants in HIV Nef. Nef is highly immunogenic, with Nef-specific CTL detectable in 95% of infected individuals(24). Escape from Nef-specific CTL during both acute(25) and chronic(3, 26) HIV infections has been documented. Additionally, Nef is unaffected
by antiretroviral treatment and is not a target for neutralizing antibodies. Therefore, Nef provides 
an excellent target for examining CTL escape with minimal confounding effects from other 
selective pressures. Nef is also a tractable target for sequencing, since the entire reading frame 
is approximately 200 amino acids.

**Strategy for exploring the latent reservoir in HIV+ volunteers**

We are currently enrolling up to 25 HIV+ participants per year, all of whom will be re-
cruted through the UW-Madison Hospitals and Clinics. We have already collected samples 
from six HIV+ individuals and are currently enrolling participants at the rate of 1-2 per 
month. 24 mL of blood is being collected from each patient. The whole blood is being separated 
into lymphocyte and plasma fractions. We will recover archival viruses from the latent reservoir using 
the optimal strategy discovered in the SIV studies (Specific Aim 1) and compare the sequences 
of these reservoir viruses to the contemporary circulating virus in the blood plasma.

We have successfully piloted the core experimental methods necessary for these 
experiments. Activated and resting lymphocyte populations have been differentiated by multi-
parametric flow cytometry and the sequences of the predominant, circulating nef sequences from 
plasma have been determined for the first six HIV+ volunteers (Figure 4). Once we obtain the lymph-
ocyte subpopulation containing the latent reservoir, cloning individual viruses from the latent 
reservoir should be straightforward.

We expect to sequence approximately 24 clones per reservoir, but this number may increase 
if we detect a bias towards contemporary sequences in the SIV studies. The sequences of the 
clones from the reservoir will be aligned to the predominant circulating sequence in the plasma. We 
will define sequences with the greatest divergence from the plasma sequence as the putative ear-
liest sequences. The plausibility of these assignments will be verified by the identification of 
subsets of clones with intermediate mutations.

Once we have obtained an alignment showing differences between the putative early se-
quins and the contemporary plasma sequence, we will examine Nef for imprinting by cellular 
immune responses. CTL responses that select viral escape mutants during early infection often 
affect more than one residue within an epitope (e.g., Tat SL8; Figure 2). Therefore, we will annotate 
regions where two or more amino acid substitutions accumulate within a 10 amino acid window as 
possible CTL epitopes. Putative CTL epitopes will be screened against the predicted peptide-
binding motifs for each individual's HLA class I genotypes. Viral mutation within regions predicted
as CTL epitopes for a given individual’s HLA genetics provides strong circumstantial evidence that the observed variation is CTL mediated. We will also examine the HIV Immunology Database to investigate the possibility that CTL responses against putative epitopes have already been described. For at least five candidate epitopes that are identified through this process, we will attempt to expand residual memory CTL in vitro and test their antiviral activity to validate our approach to identifying CTL responses that are marginalized by escape during early infection.

**Summary**

This project seeks to leverage technologies developed for examination of HIV drug resistance to improve the identification of effective HIV-specific CTL responses. Taking advantage of the expertise of both basic researchers and clinicians at UW Hospitals and Clinics, this project interfaces the experimental malleability of the SIV-infected rhesus macaque model for AIDS with the relevance to human disease that only studies of HIV can provide. Successful completion of this project will provide new insights into HIV immunity and will strengthen the translational HIV/AIDS research program at UW-Madison.
References


