Research Plan

A. Specific Aims

A large number of children with perinatally-acquired HIV infection are now surviving into adolescence and adulthood. Cellular chemokine and chemokine receptor gene mutations, viral *nef* gene mutations, certain HLA types, and other host and virus properties have been associated with slow progression of illness in some infected children. Many have also experienced beneficial effects from highly active antiretroviral therapy (HAART), in whom suppression of viremia has been followed by correction of T cell abnormalities, often due to thymus-dependent regeneration. Recent studies involving infected adults suggest that beneficial effects may be seen even if viremia continues, if HAART selects for HIV variants with impaired replication in thymic tissue.

However, the future of these perinatally infected adolescents remains uncertain. For many, delayed diagnosis of HIV infection led to delayed initiation of antiretroviral therapy until significant immunological abnormalities were already present. Many were also treated with inferior regimens prior to the development of HAART for children in the late 1990's. Thus, most have experienced high level HIV viremia throughout the period of post-natal immunological development.

We hypothesize that prolonged and poorly controlled HIV infection throughout childhood may cause premature immunological senescence, perhaps exacerbated by physiological thymic involution in childhood and adolescence. We propose a longitudinal study to examine thymopoiesis, HIV-specific *and recall* immune responses, and virologic characteristics of perinatally infected adolescents. Our investigation will involve a large cohort of perinatally infected adolescents, and two age-matched control groups: adolescents who acquired HIV infection via adult behaviors (sexual contact and illicit drug use), and seronegative adolescents. The specific aims are:

1. **To compare quantitative parameters of thymopoiesis and *T cell turnover* from adolescents/young adults with perinatal HIV infection (PI-A) with those from age-matched seronegative control subjects (SN-A), and youths with HIV infection acquired via recent adult behaviors (AB-A).**
   Measurement of T cell receptor excision circles (TREC), flow cytometric measurements of naive lymphocyte subsets, volumetric radiographic imaging of the thymus, and *in vivo* metabolic labeling of T cells will be used to provide a comprehensive assessment of thymic activity, *T cell turnover*, and the characteristics of peripheral T cell subsets. These measures will be correlated with clinical and laboratory parameters of pubertal development to determine if puberty exacerbates the impact of HIV infection on thymic involution.
2. **To evaluate the impact of viral factors on thymopoiesis of HIV infected adolescents.**
   HIV will be isolated from PI-A and AB-A adolescents with long-term suppression of viremia, and from PI-A with discordant treatment responses (stable T cell counts, with detectable plasma HIV RNA). The replication of these isolates will be examined in cultures of lymphocytes, thymocytes and thymic organ cultures. We will determine if drug resistance or *nef* gene mutations are modulating the virulence of the virus variants present in these adolescents.
3. **To examine the breadth of cellular immune responses of perinatally infected adolescents.**
   Using pools of overlapping synthetic peptides to represent the complete HIV protein repertoire, we will thoroughly map the breadth and depth of HIV specific CTL responses. *Elispot and lymphocyte proliferation assays will be used to quantify recall responses to Candida, Epstein Barr Virus, and influenza antigens. The T cell receptor Vb repertoire of influenza and HIV specific CTL will be examined, to determine if perinatal HIV infection results in persistent perturbations of the T cell receptor repertoire, despite prolonged antiretroviral therapy.*

With these studies we hope to understand better the immunological status and prognosis of long-term survivors of perinatal HIV, and to identify possible therapeutic strategies to promote a normal, healthy lifespan for this growing population of infected youths.

B. Background and Significance

Long term survival after HIV perinatal infection.

Most cases of pediatric HIV-1 infection result from perinatal infection, occurring either *in utero* or at the time of delivery. Other cases are acquired postnatally via transfusion or breast-feeding. Regardless of the means by which infection is acquired, untreated pediatric HIV infection is generally followed by the development of symptomatic disease in the first year of life, and the development of AIDS in as many as

50% of children by 5 years of age [1, 2] . In the pre-HAART era, the median and mean survival times for perinatally infected children were 8.0 and 9.4 years, respectively [2, 3] . However, survival into adolescence is now occurring in many cases. The number of these perinatally infected adolescents is unclear, but the most recent HIV/AIDS Surveillance Report [4] notes 180 people who acquired HIV infection perinatally who have had AIDS diagnosed after age 13. It is likely that a much larger number of children were diagnosed with AIDS prior to age 13, and are now surviving into adolescence because of improvements in preventative care, and the advent of highly active antiretroviral therapy (HAART). In concert with the marked decrease in perinatal transmission seen in the last seven years, this increasingly large population of perinatally infected adolescents is changing the face of Pediatric HIV/AIDS in the United States.

Survival into adolescence is likely to be attributable to a combination of viral, host, and treatment factors. Chemokine and chemokine receptor gene polymorphisms, HLA type, and mutation of the viral *nef* gene are potentially important variables [5] . However, CCR5 deletions are uncommon in non-Caucasian populations that are most affected by the HIV epidemic [5, 6] , and large deletions in *nef* appear to be rare. There have been two small reports [7, 8] in which mutations of the *nef* gene were found in 8 long-term survivors of perinatal infection. In these reports, two patients had large *nef* gene deletions, and had strikingly mild disease. One was asymptomatic and on no antiretroviral therapy at 10 years of age, and did not have detectable plasma HIV RNA. The other was 12 years of age and had only moderately symptomatic disease (CDC class B). Missense mutations and small deletions of *nef* were found in some of the others. In contrast, most long-term surviving children have much more advanced disease than the subjects described in these reports. Nielsen et al [9] reported a multicenter study of pediatric long-term survivors, defined to be 8 or more years of age. Only 31/143 (21)% of children with maternally-derived HIV infection and 9/54 (17%) with transfusion acquired infection had $\geq$ 500 CD4+ T cells and no prior AIDS defining conditions. Thus, in most cases, progressive immunodeficiency and AIDS are seen in adolescents who acquired HIV infection perinatally or via transfusion in early childhood, suggesting that these individuals are infected with fully pathogenic HIV variants.

Immunological changes associated with HAART.

The accumulated experience with HAART in adults has shown us that the prolonged suppression of HIV viremia to “undetectable” levels will often (though not invariably) arrest disease progression and bring about significant immunological restoration. Initially, the marked increase in CD4+ T cells seen with potent anti-retroviral agents was attributed to the reduction in virus-induced destruction or clearance of cells [10, 11] . Later reports made it clear that redistribution of lymphocytes from lymphoid organs accounted for much of the initial increase in peripheral blood T lymphocytes [12-14] . Subsequently, the qualitative and quantitative changes seen in response to HAART were better characterized. A triphasic pattern of immune reconstitution was described by Autran et al. in studies of adults. An early rise of memory CD4+ T cells was soon followed by improved T cell proliferation responses to recall antigens, and, finally, a late rise in putative naïve CD4+ T cells (CD45RA+ CD62L+). Functional improvements in cellular and humoral immune responses are often evident within months [15] . Similar patterns have been observed following HAART in children, although CD45RA+ T cells often increase in number during the first several months of therapy [16, 17] . Immunological improvements are less well documented during HAART in children, but include improved responses to measles immunization and proliferative responses to Candida [18-20] .

These data and the demonstration of decreased risk of opportunistic infections following prolonged HAART therapy [21, 22] have engendered optimism that suppression of HIV replication may be followed by at least partial restoration of normal immunity. However, it is clear that quantitative and functional immunological abnormalities generally persist. T cell counts remain below the normal range in most adults, and opportunistic infections continue to occur in some individuals. At a more subtle level, the T cell receptor repertoire continues to show marked perturbation even after long periods of successful HAART. This has been assessed in a number of ways [23-25, 25a], particularly by using RT-PCR to examine the size distribution of sequences in the third coding complementarity determining region 3 (CDR 3) of the T cell receptor (TCR) β chain. Insertion and removal of nucleotides during TCR recombination normally leads to a Gaussian distribution of the size of amplicons resulting from RT-PCR amplification of Vβ mRNAs with primers that flank junction sites. Skewed distributions in CDR3 size pattern detected by this “spectratyping” are seen in chronic HIV infection, and often persist despite successful HAART [23-25] .

Persistent immunological defects may be more extreme following prolonged HIV infection in childhood. HIV infection in adults occurs after immunological development is complete, and substantial immunological experience with common pathogens has occurred. Perinatal HIV infection in children is associated with high viral RNA levels throughout the first several years of life, perhaps leading to a disruption of immunological ontogeny, interfering with the acquisition of protective responses to CMV, toxoplasmosis, and other microorganisms [26, 27] . Moreover, HIV has been associated with accelerated thymic involution, which could be exacerbated by the sustained high-level viremia characteristic of early childhood. Indeed, an immunophenotype consistent with DiGeorge anomaly has been described in children with rapid progression of disease [28, 29] , *and data have recently emerged suggesting that thymic output is impaired by HIV replication* [29a, 29b]. The mechanism (s) underlying thymic injury by HIV are unclear, but the virus might directly kill thymocytes, or disrupt the microenvironment of thymic dendritic cells and epithelial cells required for normal thymopoiesis [30, 30a] . Although data from the SCID-hu mouse model indicate that the thymic epithelial compartment may remain functional long after depletion of thymocytes has occurred [31] , there may be limits to this: age dependent difference in responses to HAART have been noted. Some studies indicate larger increases in CD4+ T cell counts are seen in younger compared to older adults [32] and in children compared to adults [33] . HAART may be less beneficial in perinatally infected adolescents, who have had the longest exposure to potentially injurious effects of HIV replication.

Patterns and parameters of thymopoiesis.

The potential impact of perinatal HIV infection on the future survival of those who have survived into adolescence must be considered in light of recent developments in the understanding of thymopoiesis.

HISTOPATHOLOGY: Until approximately twenty years ago, the thymus was thought to enlarge in childhood, and then involute during puberty under the influence of growth hormone and sex steroids. More recent studies of anatomy and physiology of the normal thymus have called this notion into question [30, 34] . Careful histological analysis of tissue revealed the thymus to be a chimeric organ containing epithelial tissue, where thymopoiesis occurs, and non-epithelial, perivascular space (PVS). In childhood, the thymic epithelial space (TES) decreases progressively, while the perivascular space gradually enlarges [35] . During adolescence, the total amount of lymphoid tissue remains constant, owing to an increase of the lymphoid PVS. Following adolescence, the PVS decreases, and fatty atrophy of the thymus is seen. However, after accounting for the perivascular space, Steinmann demonstrated a continuous loss of the lymphoid tissue in the cortex from 1 to 40 years of age [34] , without evidence of any change in the rate of loss during or after puberty. After the age of 40, the rate of involution of the TES decreases, but loss of the tissue involved in thymopoiesis continues. Still, functional TES has been seen in centenarians, and recent reports have provided evidence of functional thymic activity in the fifth and sixth decades of life [36, 37] . While the thymus does not normally decrease in total volume with aging, HIV infection has been associated with a decrease in thymic volume, and histological evidence of premature atrophy, with decrease in TES, and an increase in the PVS. This effect may be a direct effect of HIV replication: HIV infected cells have been found in both the PVS and the TES [30] . The recent detection of infected naïve peripheral CD4 T cells is also consistent with this possibility [38, 39] .

If there is an effect of puberty on the rate of involution of the thymus, it does not appear to be readily detected in those not infected by HIV. However, delayed onset of puberty has been often seen in pediatric long-term survivors of HIV infection [40] , and castration prevents post-pubertal thymic involution in mice [41, 42] . It is conceivable that delayed puberty will have an effect on the rate of thymic involution of perinatally infected adolescents. If higher levels of testosterone and other androgens promote loss of functional tissue, delayed puberty may actually be beneficial, from a teleological perspective, in the face of ongoing HIV replication.

RADIOGRAPHIC IMAGING: Until recently, non-invasive measures of the amount of functional thymus did not exist. An ideal laboratory method for the evaluation of HIV infected individuals would *quantify current T cell production and the amount of tissue present capable of supporting T cell differentiation, to provide an estimate of thymic activity and its functional reserve (by analogy to other physiological systems).*

Computed tomography has been used to examine the size of the thymus in adult lacking HIV infection, corroborating post-mortem studies that found no change in the size of the thymus with age [43] . Clearly, radiographic imaging has significant limitations as a tool to evaluate the capacity for thymus-

dependent regeneration of T cell populations during HAART (or after cytotoxic chemotherapy or bone marrow transplantation). However, McCune and his collaborators found a correlation between thymic mass (any residual tissue not replaced by fat scored as an ordinal variable on a five point scale) and CD4 T cell number in HIV infected adults who were 20 to 59 years of age [44] . They also found a correlation between these estimates of the amount of thymic tissue, and increased thymopoiesis during HAART [45] . Similarly, Vigano et al used magnetic resonance imaging to monitor changes in thymic volume during HAART in a cohort of children with a mean age of 9.8 years at entry [46] . The thymus was markedly diminished in volume in HIV infected children with Class C disease. A rise in CD45RA+CD62L+ CD4+ T cells during HAART and was statistically associated with a change in thymic volume. Thus, the size of the thymus, as determined by radiographic imaging, has correlated with an increase in naïve T cells during HAART of both adults and children. *Perinatally-infected adolescents have not been specifically examined.*

MEASUREMENT OF T CELL RECEPTOR EXCISION CIRCLES (TREC): Until recently, no methods existed to identify and quantify lymphocytes that have recently emigrated from the thymus. In chickens, lymphocytes may be identified as recent thymic emigrants (RTE) with the monoclonal antibody chT1 [47, 48] . No comparable cell surface marker exists for humans or other mammals, but methods to quantify RTE have been developed based on the use of quantitative PCR measurements of circular DNA molecules generated during excisional rearrangement at the T cell receptor alpha [37, 49] or beta [50] chain loci. Variations of a PCR assay described by Douek et al [37] are used to quantify circular DNA molecules (termed signal joint TREC (sjTREC)) produced in approximately 70% of the cells with excision of the δ locus during maturation of thymocytes into $\alpha\beta$ T cells [51-53] . In control subjects, Douek et al found a nearly linear inverse correlation between age and TREC number in CD4+ and CD8+ T cells. These findings are in accord with the histological studies described above suggesting a gradual, progressive, diminution of the thymic epithelial space throughout the first four decades of life, without evidence of marked changes during puberty. In accord with these data, we have also found that TREC values are very stable in serial samples from HIV seronegative adolescents followed for up to 48 months (see Preliminary Studies below).

In agreement with several studies from adults, decreased TREC numbers are seen in chronically HIV infected children, and there appear to be age-dependent differences in responses to HAART: the increase in TREC seen with HAART was greater in infants than in children with a median age of nine years [54] . Hazenberg et al have recently questioned the utility of TREC quantitation as a parameter of thymic output, noting that proliferation of naïve T cell populations may explain the drop in TREC seen after HIV infection has occurred [55] . However, this interpretation been disputed, as it is based on the measurement of TREC in measurement of cells identified as "naïve" by expression of CD27 and lacking CD45RO. As noted by Grossman and Paul, these markers would also be present in activated cells in transition to the expression of markers identifying them as memory T cells[56] . *Exclusion of these transitional cells during analysis has reafffirmed the utility of TREC measurements as a parameter of thymic output [29a].*

IN VIVO LABELLING TO ASSESS T CELL PRODUCTION, SURVIVAL, AND TURNOVER: The *survival* of T cells produced during HAART could also be influenced by the duration of previously uncontrolled HIV infection. Recent data suggest that maintenance of naïve and memory cells requires a supportive environment in the periphery, which may be distinct for each population [57] . While the signals necessary for the maintenance of the naïve T cells are still being defined, current data suggest that naïve CD4+ T cells are maintained without proliferation by contact with cells bearing MHC class II molecules [58] . Presumably, this represents the peripheral lymphoid mass, implying that HAART may need to bring about substantial normalization of lymph node architecture before immunological reconstitution is optimal. The chances for reconstruction of this microenvironment may be lower with longer periods of uncontrolled HIV replication after perinatal infection.

Directly measuring T cell dynamics in vivo may make it possible to determine if differences in T cell survival play a role in the clinical heterogeneity of long-term survivors of perinatal HIV infection. Hellerstein, McCune and their collaborators have shown that this is possible, by developing methods to determine rates of T cell turnover and cellular half-life [45, 59] . They administered prolonged infusions of glucose labeled with deuterium, a non-–radioactive stable isotope, to uninfected and HIV-1 infected adults. Using gas chromatography-mass spectrometry, the fractional replacement of deuterium for hydrogen atoms was measured, allowing them to calculate the rate of turnover of T cells in the peripheral blood, and to estimate the half-lives of CD4 and CD8 T cells. In untreated HIV infected subjects, T cells had decreased half-lives,

and no compensatory increase in T cell production. HIV treatment was associated with an increase in the absolute production rate of peripheral blood T cells, and normal half lives and production rates were restored after 12 to 36 months of HAART. In Preliminary Studies performed with the assistance of Dr. Hellerstein, we have shown that these methods can be successfully applied to newborn macaques weighing *less than 1 kilogram*.

Deeks et al, have also recently helped explain the phenomenon of so-called "discordant responses" to HAART, in which higher CD4 T cell counts and peripheral blood TREC concentrations are measured in the face of high plasma HIV RNA concentrations [60] . This appears to be due in part to diminished fitness of the virus, resulting from *pol* gene mutations selected and maintained by the pressure of HAART. Specifically, they showed that HIV with mutations that confer decreased susceptibility to protease inhibitors replicated poorly in thymic organ cultures. Removal of HAART was deleterious for the individuals they studied, who experienced a decrease in T cell counts within two to four weeks, which correlated with the re-emergence of circulating virus with increased susceptibility (decreased resistance) to reverse transcriptase and protease inhibitors. *Moreover, T cell turnover rates were near normal in those with discordant treatment responses, and several-fold below those seen in untreated subjects [60a].* These data suggest that maintenance of a drug resistant genotype and phenotype may be an acceptable therapeutic goal for selected patients in whom HAART fails to control HIV replication. This is likely to be a fruitful area of investigation in the study of perinatally infected adolescents, who often have already been treated with a series of therapeutic regimens that failed to suppress HIV viremia.

Quantitation of CTL responses

The rate of progression of HIV related disease in untreated individuals is clearly related to the level of plasma viremia that becomes established after acute infection. By analogy to homeostatic physiologic mechanisms, this has been referred to as the viral "set-point", which appears to be linked to the magnitude of CD8 responses to HIV antigens [61] . Other studies involving adults have shown that slow progression of disease is correlated with the presence of potent CTL and CD4+ helper responses [15, 62-64] .

Until recently, the mapping of CTL responses has been extremely labor intensive and technically challenging. Whole peripheral blood mononuclear cells (PBMC) from infected persons were randomly cloned at limiting dilution, followed by screening for HIV-specific cytolytic activity by chromium release using recombinant vaccinia. Alternatively, whole PBMC were specifically stimulated with recombinant vaccinia-infected autologous B cells (to enrich for HIV-1-specific CTL), then purified by limiting dilution cloning. More recently, the Elispot assay has been increasingly used to map the breadth and magnitude of HIV-1 specific cellular immune responses [65, 66] . Fine-mapping of CTL responses is possible, due to the great sensitivity of this technique, which can identify cytokine release from individual cells. Because synthetic peptides slightly larger than the optimal epitope can be bound to class I MHC molecules when added exogenously, mapping of CTL responses within bulk uncloned PBMC can be accomplished using overlapping synthetic peptides covering the protein of interest.

This approach has been recently applied to the study of CTL responses in acute and chronic HIV. For example, Goulder et al. used 290 overlapping peptides representing Gag, nef, RT, gp41, gp120, Tat and Rev , and 130 additional peptides represent previously known epitopes to map CTL responses in 11 subjects with early infection [66] . In two subjects, response to a common HLA-A*0201 restricted epitope were not initially detected, showing that these responses evolve, and revealing the limitations of targeting mapping based on HLA type. These and other recent studies demonstrate that 1) cellular immune responses can evolve in a rapid fashion, 2) cannot be dependably predicted on the basis of MHC restriction [67] , and 3) can be thoroughly examined and quantified without necessarily knowing the amino acid sequences of autologous virus sequences, or an individual's HLA type.

Summary.

Detailed studies of the interplay between HIV infection, thymic output (thymopoiesis), and antiretroviral therapy, have been critical to recent advances in the understanding of the pathogenesis of HIV infection and AIDS. The urgency to better understand the effects of HIV on thymopoiesis is evident when we consider that HAART has made survival into adulthood quite likely for the majority of infected infants and children in the United States and other countries where intensive antiretroviral therapy is available and accessible. Comprehensive studies of thymopoiesis, and characteristics of HIV that impair it may guide our efforts to preserve and improved immunological functions in HIV infected adolescents. PCR methods to quantify recent thymic emigrants, and *in vivo* labeling methods to track the fate of these cells,

have made it possible to more comprehensively examine the mechanistic underpinnings of T cell depletion in pediatric HIV infection. For the first time, methods now exist to quantify with some accuracy the production, function, and clearance of T cells.

We do not know what combination of viral, host, and treatment factors has allowed some infants to survive into adolescence after perinatal HIV infection, and can presently only speculate what the future holds in store for them. Taking scientific advantage of the large number of perinatally infected adolescents and young adults in the Los Angeles area, we are proposing studies to examine the balance between the pathogenic properties of HIV, the suppressive and selective power of antiretroviral therapy, and the regenerative capacity of the immune system that exists in these individuals.

C. Preliminary Studies

Using support from an Elizabeth Glaser Pediatric Scientist Award to Dr. Krogstad, we have performed an initial cross sectional comparison of T cell subsets and TREC values in long term surviving adolescents and young adults (LTS-A), age-matched seronegative adolescents (SN-A), and youth who have acquired infection sometime during adolescence via sexual contact (Adult behavior infected (AB-A). Thus far, nearly all of the subjects enrolled are seronegative and HIV infected adolescents who are followed in clinics at Children's Hospital Los Angeles under the supervision of Drs. Marvin Belzer and Joseph Church. Demographic and clinical characteristics of the adolescents studied thus far are summarized in Table 1.

Table 1	**LTS-A** N= 16	**AB-A** N= 14	**SN-A** N= 7
Age: mean (standard deviation)	16.1 (2.1)	19.5 (1.7)	16.7 (1.8)
Gender (F/M)	9F/7M	3F/11M	5F/2M
CDC Classification N	1	0	-
A	0	11	-
B	5	3	-
C	10	0	-
Mean Viral Load (copies/ml)	16770	12318	-
Number of subjects with HIV RNA≤ 50 copies/ml	2	5	-
T cells			
CD4+ (cells/μL) mean (median)	367 (207)	669 (476)	996 (836)
%CD4 + mean (median)	17.9 (16)	27.5 (27.5)	43.5 (44.0)
% Naïve CD4+	52.9	36.5	50.1
CD8+ (cells/μL) mean (median)	914 (949)	768 (666)	478 (476)

Data are shown from LTS-A who acquired infection perinatally (n=14), or via transfusion as newborns (n=2). The LTS-A and seronegative adolescents have a mean age of 16 years; the AB-A are somewhat older in age (20 years). (Of note, the majority of the LTS-A children studied are on therapy, usually consisting of regimens containing 3 or four antiretroviral agents, *while most of the AB-A examined are not receiving therapy. Entry to this pilot study was not restricted or stratified on the basis of antiretroviral therapy. For the studies proposed in this resubmission, we will take care to compare parameters of thymopoiesis only between subjects carefully matched for level of viremia, and type of therapy. (See "Recruitment of subjects" heading under Section D, Experimental design)).*

Using PCR primers described by others [68] , we amplified the site of the Δ32CCR5 mutation; all 15 of the LTS-A subjects examined to date lacked this mutation (data not shown). T cell immunophenotyping was performed at the time of enrollment using whole blood staining with FITC, PE, and APC labeled CD3, CD4, CD8, CD45RA, and CD62L antibodies. The analysis was performed in the UCLA CFAR laboratory. CD4 and CD8 quantitation was available for all, and CD45RA + C262L+cell data were available from 10 LTS, 13 AB-A, and 6 SN-A. The CD4+ T cell percentage and number were approximately two fold lower than the AB-A perhaps as a consequence of therapy. Interestingly, the CD45RA+ CD62L+ fraction of CD4+ lymphocytes was greater in the PI-A, compared to the AB-I. Access to such cohorts of infected and uninfected adolescents will make it possible to assess the impact of long-term survival with HIV infection on thymopoiesis.

TREC assay

In preliminary studies we have quantitated TREC by real-time polymerase chain reaction analysis, using the 5' nuclease (TaqMan) assay), using primers and conditions described by Douek et al [69] . The primers used amplify a nucleotide sequence that flanks the signal joint formed during excisional rearrangement of the TCR α locus. A standard curve was established by amplifying in parallel reactions serial dilutions of a plasmid containing signal joint TREC fragment (a gift of D. Douek) (range: 25 to 10^6 copies/ reaction, diluted in salmon sperm DNA carrier and lysis buffer). TREC values for test samples were calculated with the software provided with the ABI Prism 7700 sequence detector system. Each sample was run in triplicate and mean TREC values were used for data analysis. (Figure 1). In a similar fashion, we quantified cellular DNA by amplifying CCR5 DNA sequences in duplicate reactions from cellular lysates, using DNA extracted from peripheral blood lymphocytes (PBMC) of normal donors by simple protease digestion. These results are reported as TRECs/10^6 cells, using the estimate of 8 micrograms of DNA/million PBMC.

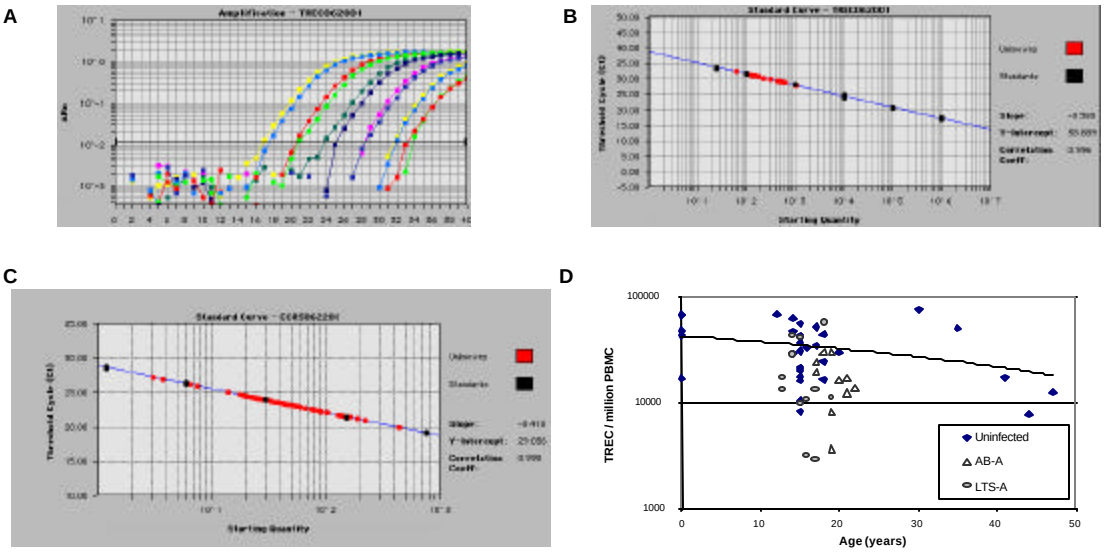


Figure 1. Quantitation of TREC sequences using TaqMan PCR. Signal Joint TREC derived from excisional rearrangement of the TCRA locus were detected by Taqman PCR. A standard curve was generated by amplifying serially diluted plasmid signal joint sequences. Panels A , B: TREC amplification plot and standard curve.
Panel C: Standard curve from amplification of CCR5 sequences in known amounts of PBMC DNA.
Panel D. TREC values from amplification of samples from uninfected subjects (cord blood samples, adolescent and adult volunteers), and HIV infected long terms survivors (LTS-A) and adolescents/adults infected by adult behavior (AB-A).

During the process of assay standardization, we compared TREC from cord blood lymphocytes and from PBMC samples from healthy subjects. TREC assay results in a subset of the latter group of patients and healthy control subjects were comparable with results obtained with the same samples by Zhang et al [49] . Typical amplification plots and standard curves for the CCR5 and TREC assays are shown in Figure 1, panels A to C. In panel D, preliminary data are shown from the analysis of PBMC from cord blood samples of normal infants, uninfected adolescents (including the SN-A group above) and adults, and from the two groups LTS-A and AB-A described above. There was a linear decrease in TREC values with increasing age, and the median TREC value was lower in LTS adolescents compared to adolescents with HIV infection acquired by adult behaviors. TREC values of both LTS group and the AB-A were significantly lower than the control group of SN-A ($p<0.007$ and 0.006 by T test and Wilcoxin rank sum test). TREC values for 75% of the LTS-A were below the median value of those of the SN-A. These preliminary data indicate are consistent with reports showing decreased TREC values in HIV infected children and adults [37, 54] , and appear to be lower in LTS than AB-A.

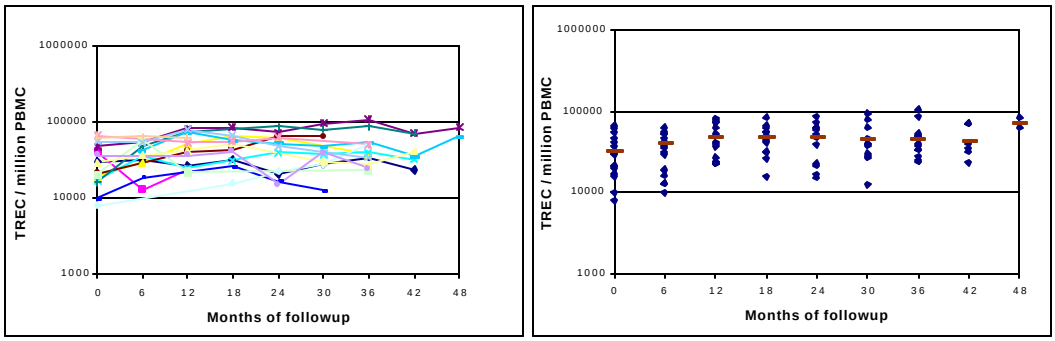


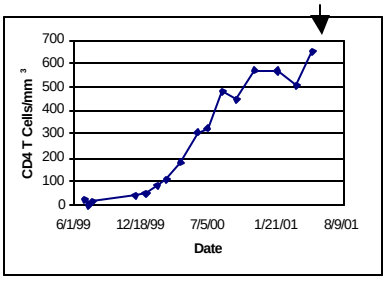
Figure 2. TREC values from control subjects enrolled in the REACH project. Left panel: longitudinal data from 16 subjects followed for up to 48 months. Right panel: Same data as left panel redrawn to show mean (horizontal bar) and distribution of values.

We have used this assay to examine TREC in samples from 16 seronegative subjects enrolled as controls in the REACH study, a longitudinal study of HIV pathogenesis in adolescents conducted at 16 sites in the United States [70] . These subjects were followed for periods of up to 48 months. The TREC values obtained were very stable, varying less than three fold (Figure 2). *Linear regression was performed on the aggregated TREC values over time. The trend was not significantly different from zero (p=0.25),* consistent with morphometric and histological studies [34] that have indicated that the amount of the functional thymic epithelial tissue is relatively stable over such a period.

Volumetric computed tomography of the thymus

Radiographic imaging has been used to examine the size of the thymus in HIV infected pre-pubertal children and adults, using scoring scales [44] , or crude approximations of volume afforded by calculations based on measurements of approximate length in 3 planes [71] . The size of the thymus is difficulty to assess with these methods. The thymus is an irregularly shaped, generally bi-lobed organ [72] . Asymmetry of the lobes is common, and the thymus in some cases extends into the posterior mediastinum.

As an alternative, we have prepared a protocol to use using rapid (spiral) computed tomography (CT) to calculate the actual volume of the thymus of HIV infected and control subjects. An example is shown of the evaluation of an 18 year old (11-01) who had acquired HIV infection by transfusion after premature delivery. He had never received any antiretroviral therapy or prophylaxis against opportunistic infection, and presented to UCLA Medical Center in June, 1999 with disseminated *Mycobacterium avium* infection. HAART was initiated in June, 1999 with the combination of Zidovudine, nelfinavir, and lamivudine. His HIV plasma HIV RNA dropped rapidly from its initial value of over 1,000,000 copies/ml and became undetectable within 3 months of initiating HAART. Approximately six months later, his T cell counts began to increase steadily. Twenty-four months after beginning therapy, his CD4+ T cell count was more than 500 cells/μL, and 62% of his CD4+ T cells were of the CD45RA+CD62L+ phenotype (Figure 3).



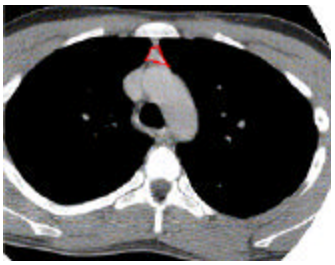


Figure 3. Left Panel: CD4+ T cell counts over time following initiation of HAART in June 2000 of a 16 year old adolescent who acquired HIV infection via a perinatal transfusion after premature birth. **Right panel.** CT of chest was performed at 18 years of age, after 24 months of HAART (marked by arrow in left panel). Chest was scanned from thoracic inlet to mid-thorax with 3 mm collimation.

At that time, TREC positive cells were present in his peripheral blood as 56,000 TREC/million PBMC (higher than the mean value found in HIV seronegative adolescents; see above), providing additional evidence of resurgence of thymopoiesis. Using a scanning protocol approved by the UCLA Office for the Protection of Research Subjects, after review by the Medical Radiation Safety Committee, we examined his chest by CT. His thymus was markedly diminished in size, compared to most adolescents, but showed little evidence of fat replacement. The volume of the thymus was measured from CT data in the Quantitative Image Analysis Lab (UCLA Department of Radiology), using custom software that applies a parametric model of anatomy to identify the boundaries of the thymus in three-dimensions (as a guide to image segmentation) and then sums the volumes of the included voxels [73-75] . Despite evidence of active thymopoiesis, the volume of his thymus was extremely small (5.8 ml), compared to the expected volume of about 20 ml [34] . This imaging approach provides a more accurate measure of existing thymic

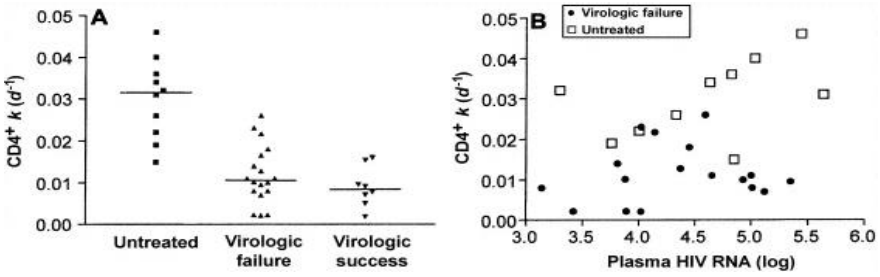


Figure 4. Fractional replacement rate (a measure of T cell turnover) in HIV infected adults. A, Fractional replacement rates *(k)* of $CD4^+$ T cells are shown for 3 groups of HIV infected adults(untreated, treated patients with persistent plasma HIV RNA (≥ 2500 copies/ ml in last 6 months) (discordant responses), and virologic success(long-term suppression of HIV RNA to <50 copies per ml for at least the preceding 6 months)). **B**, *k* vs. the absolute level of viremia (log_{10} copies/ mL) for the 2 treatment groups

tissue than a subjective visual assessment, and provides quantitative parameter that we will incorporate into a comprehensive assessment of thymopoiesis in other long-term survivors of perinatal HIV infection.

In vivo labeling of lymphocyte turnover in humans and newborn rhesus macaques

T cell subsets, TREC values, and radiographic measures of thymic volume are static parameters that cannot provide information about the dynamics of lymphocyte production, differentiation, and life-span. Our consultant, Marc Hellerstein, and his collaborators, have developed in vivo labeling methods that allow the turnover and absolute rates of lymphocyte production to be calculated [29b,45,59-60a]. In brief, subjects are infused for 1 to 7 days with glucose that has been labeled by substitution of the stable isotope deuterium for several of the hydrogen atoms. At various times after this labeling period, blood is drawn, and the DNA from purified lymphocytes is subjected to enzymatic hydrolysis and gas chromatography-mass spectrometry (GCMS) analysis. Knowing the period of labelling allows for the calculation of fractional replacement (k) and absolute production rates per day for T cells. These data have provided crucial insight into the nature of HIV associated CD4+ lymphocytopenia, which has been attributed to cytopathic effects of HIV, the deleterious effects of immune activation, inhibition of thymopoiesis [30a]. In particular, they have demonstrated recently that HAART is associated with increased T cell production, and normalization of T cell turnover rates, and that this may be seen in those with "discordant treatment responses" i.e. those in whom CD4 counts increase with HAART, despite ongoing plasma viremia (See figure 4, from Deeks et al 2002 (Provided in Appendix)).

Fractional Replacement rate constants k (day^{-1})

	CD4	*CD8*
Animal # 32644	0.024	0.042
Animal # 32653	0.028	0.042
Humans*	0.0085	0.005

Values from total CD4 and CD8 T cells.
* Human values are from McCune et al (2000)

In collaboration with Drs. Marta Marthas at UC Davis and Dr. Hellerstein, pilot experiments were performed with macaques to test the feasibility of using in vivo labeling techniques to measure the turnover of T lymphocytes in infants and other pediatric subjects. Formula with glucose containing the stable isotope deuterium as the sole carbohydrate source was given to neonatal rhesus macaques for 24 hours. Seven and 14 days later, PBMC samples were obtained for DNA preparation and GCMS analysis. Excellent label incorporation was detected in this initial experiment, but the failure to obtain samples of blood at the end of the labeling period precluded calculation of turnover rates. In a second experiment, two newborn rhesus macaques weighing slightly more than 600 grams each were fed formula containing 35% D_20 for seven days. CD4 and CD8 T cell subsets were purified by fluorescence activated cell sorting. The DNA was purified and analyzed, as previously described [59] . The calculated T cell turnover rates from two animals that were several fold higher than those measured in the adult humans studied described in studies performed by Drs. Hellerstein, and McCune and their collaborators.

These experiments allowed us to test the feasibility of oral labeling for human infants, and to establish protocols for handling small PBMC samples. They also demonstrate the sensitivity of using long periods of labeling with D_20. This becomes highly incorporated into cellular DNA, allowing the rate of production of naïve CD4 T cells to be quantified.

Mapping of HIV-1-specific CTL Responses

ELISpot assays are increasingly being used to map cellular immune responses to HIV and other viral agents. The use of these assays, which are rapid, highly quantitative, and technically straightforward, has revolutionized our ability to define and measure CTL responses. Through single cell sensitive measurement of interferon gamma (IFN-g) production in response to exogenously added peptides, ELISpot assays can provide relatively simple qualitative and

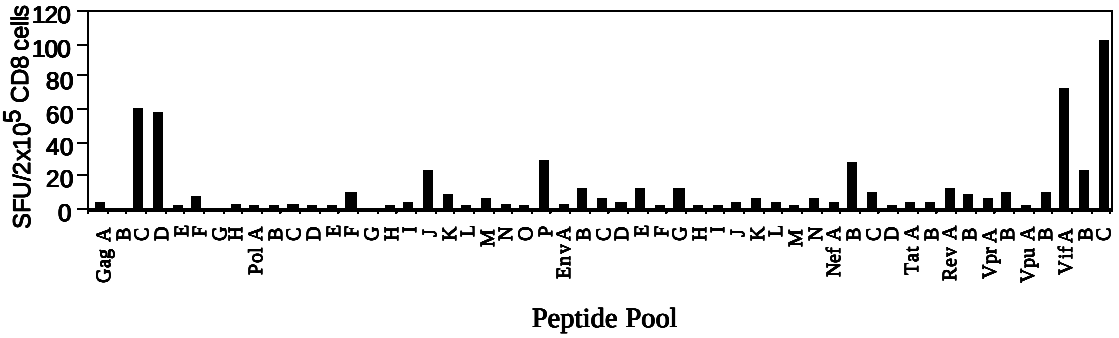


Figure 5. Initial screening for CTL responses using peptide pools. Bulk CD8+ PBMC from an HIV-infected long term nonprogressor were screened in an ELISpot assay using overlapping peptides (described above). The peptides were used in pools of 16 or less. As depicted, responses were noted in pools from several proteins. in pools of 16 or less. As depicted, responses were noted in pools from

quantitative mapping of CTL responses in HIV infected individuals. Dr. Yang's laboratory has established the working conditions to define and quantitate HIV-1-specific CTL responses using a library of overlapping peptide sequences spanning each viral pre-protein: Gag, Pol, Env, Tat, Rev, Nef, Vpr, Vpu, Vif. These peptides have been obtained from the NIH AIDS Research and Reference Reagent Program (www.aidsreagent.org), and are each 15 amino acids overlapping by 11. They therefore contain all potential epitopes of size 12 or smaller. All peptide sequences are based upon the Consensus B sequences at the Los Alamos database, with the exception of Gag and Env, which are based on laboratory strains. Dr. Yang has recently designed and ordered the Consensus B versions of Gag and Env to complete the set available through the Repository. Phylogenetic analysis reveals that this sequence is generally much more closely related to primary isolates and common strains of HIV-1 than they are to each other (Bette Korber, personal communication), and therefore a better compromise than any reference strain available. Several HIV-1-infected individuals have already been screened by ELISpot assays. Initial screening is performed using 53 pools of 16 or less peptides each (Figure 5). After identification of pools giving responses, each of these pools is analyzed using a matrix approach (Figure 6) that consists of screening pools where each peptide is contained in exactly two pools. Examination of which matrix pools give responses reveals the identity of the responsible peptides, and these peptides can then be used individually to confirm and quantitate the results (Right panel, Figure 6).

Vif C	Pool 1	Pool 2	Pool 3	Pool 4
Pool 5	Vif 6049	Vif 6050	Vif 6051	Vif 6052
Pool 6	Vif 6053	Vif 6054	Vif 6055	Vif 6056
Pool 7	Vif 6057	Vif 6058	Vif 6059	Vif 6060
Pool 8	Vif 6061	Vif 6062	Vif 6063	

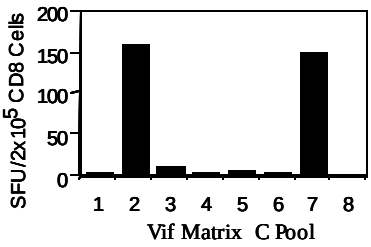


Figure 6. Matrix strategy to define recognized p eptides within recognized pools.. In this example, the 15 peptides from Vif pool C (Figure 4) were analyzed in a 4 x 4 matrix. The matrix pools of peptides contained the individual peptides listed in the column (Pools 1 to 4) or row (Pools 5 to 8) (left panel). The pools that gave responses (quantified in right panel) are highlighted, indicating that peptide Vif 6058, shared by Pools 2 and 7, contains an epitope recognized by the cells of this individual. This process was performed for all pools yielding responses in Figure 5.

If desired, fine mapping can also be used to distinguish minimal epitopes using truncated peptides, as previously reported. In cases where consecutive peptides give responses (e.g. Gag 6806 and Gag 6807, Table 2), the epitope is often located in the overlap, and this can be distinguished by checking for responses to the peptides alone and in combination, and/or fine mapping.

Protein	Peptide #	Sequence	Frequency
Gag	6783	HQAISPRTLNAWVKV	360
Gag	6798	TINEEAAEWDRLHPV	58
Gag	6806	IAGTTSTLQEQIAWM	448
Gag	6807	TSTLQEQIAWMTSNP	385
Pol	5606	WYQLEKEPIVGAETF	233
Pol	5607	EKEPIVGAETFYVDG	145
Pol	5705	RRKAKIIRDYGKQMA	303
Pol	5708	KIIRDYGKQMAGDDC	250
Env	6229	QEVELVNVTENFNMW	253
Vif	6024	VKHHMYISGKAKGWF	>1000
Vif	6025	MYISGKAKGWFYRHH	>1000
Vif	6058	PPLPSVTKLTEDRWN	>1000

Table 2. CTL responses to Vif were identified and quantified in a long term nonprogressor. Peptides identified to be recognized by matrix analysis were used individually with polyclonal CD8+ cells to confirm and quantify responses. Peptides are listed by AIDS Research and Reference Reagent Repository catalogue #. Frequency of responding cells is given per million CD8+ cells.

Mapping CTL responses using such a peptide library will be used to produce a complete assessment of the breadth and magnitude of cellular immune responses in HIV infected adolescents.

Sequence analysis of subjects enrolled in pediatric clinical trials of HAART

Dr. Krogstad was one of three lead investigators in PACTG 377, a study of multi-drug antiretroviral therapy for HIV-1 infected children at 50 sites in the U.S. and Puerto Rico (PACTG 377). One hundred and eighty one children between the ages of 4 months and 17 years were enrolled between December 1997 and September 1998 [82] (Appendix). The children were randomized to 4 treatment arms including

different combinations of d4T, lamivudine (3TC) , nevirapine(NVP), nelfinavir, or ritonavir. Subsequently, genotypic resistance testing was performed in the UCLA PACTG core laboratory, and two other centers, using plasma samples collected from these subjects. We evaluated the prevalence of drug resistance mutations in the children prior to study treatment, and the impact of those mutations on virologic treatment response. We also evaluated whether additional drug resistance mutations were selected in children who experienced protocol defined virologic failure, and whether the selection of drug resistance mutations was less common among children who received a 4-drug regimen. This represented the largest study to date of HIV-1 drug resistance in children, and the first to examine drug resistance in a large pediatric cohort in the context of a randomized, controlled trial of highly active antiretroviral therapy. One hundred ninety six samples were analyzed, including 135 baseline and 61 failure samples. Plasma volumes ranged from 0.05 to 0.5 ml and viral loads ranged from 1,084 to 3,484,991 copies/ml [83] . PCR products suitable for sequencing were obtained for 192/196 of the samples. Complete sequences for protease and reverse transcriptase were obtained for all 192 samples. For 180 samples, data was obtained from both DNA strands for the entire region analyzed. Performance of the genotyping system was similar in three laboratories, demonstrating that the genotyping system we used (Applied Biosystem ViroSeq) performs well for analysis of HIV-1 in pediatric plasma samples, including those with low volume and low viral load (Appendix).

Interestingly, children who had drug resistance mutations at baseline actually had greater reductions in viral load over time compared to children without baseline mutations [84] . Mutations associated with NVP and 3TC were frequently detected at the time of virologic failure. Furthermore, nevirapine resistance mutations at failure were more common among children receiving 3-drug nevirapine containing regimens, compared to a 4-drug combination. Children who were maintained on their initial study regimen after virologic failure accumulated mutations associated with resistance to the protease inhibitors nelfinavir and ritonavir, as well as additional mutations associated with nevirapine and lamivudine [84,84a].(Appendix)

As a quality control measure, we also performed phylogenetic analysis of HIV-1 sequences. Surprisingly, phylogenetic analysis revealed that two children, both born in the United States, were infected with non-B subtypes that are most commonly found in Africa: one with subtype D, and the other with circulating recombinant form CRF02, an A/G recombinant lineage. This was confirmed by sequencing the envelope and gag genes. These cases demonstrate mother to child transmission of non-B subtypes of HIV-1 in the US [84a].

Experience with these analytic methods will form the basis for the detection of resistance mutations of HIV variants in perinatally infected adolescents and control subjects.

Thymic organ culture.

Early infection of the thymus with the human immunodeficiency virus (HIV) may continue to the more rapid disease progression among children infected *in utero* compared to children infected intrapartum [27, 85] . In previous studies, Dr. Uittenbogaart's laboratory infected thymocytes *in vitro* with HIV-1 primary isolates that had been obtained at (or near) birth from ten children with different disease outcomes. *Well-characterized X4 (NL4-3) and R5 (JR-CSF) –tropic molecularly cloned isolates served as controls. Using our three experimental systems we previously showed that the effects of HIV infection with the X4 and R5 molecularly cloned isolates on T cell development in the thymus are similar when tested in in vitro thymocyte cultures and the in vivo SCID-hu mouse model* [86] [86a].

Our data show that HIV isolates able to replicate in the thymus and disrupt thymopoiesis were present in all infants, regardless of the timing of viral transmission and rate of disease progression. Isolates from newborns utilized CCR5, CXCR4 or both chemokine receptors to enter thymocytes. Viral expression was observed in discrete thymocyte subsets after infection with HIV isolates that use CXCR4 (X4), as well as those using CCR5 (R5). *Pediatric isolates induced a statistically significant decrease in the total $CD4^+$ population ($p=0.0001$), the $CD4^+CD8^-$ ($p=0.0001$) and $CD4^+CD8^+$ ($p=0.0001$) populations when compared with their respective mock-infected controls (see Table 3 in attached manuscript in press Pedroza et al. Appendix). Loss of CD4 expression in thymocytes infected with the different HIV isolates did not correlate with coreceptor use in the thymus, and was not increased in children with rapid rate of disease progression. Despite the wider distribution of CXCR4 than CCR5 in the thymus, no differences were found between the CXCR4-using and R5-tropic pediatric isolates in terms of the average percentage of total $CD4^+$ ($p=0.8$), $CD4^+CD8^-$ ($p=0.8$) and $CD4^+CD8^+$ ($p=0.8$) populations. These results are in contrast to the more severe depletion after infection with NL4-3 than JR-CSF (Table*

3 in attached manuscript Pedroza et al.). However, some R5-tropic pediatric isolates caused greater loss of CD4 expression in thymocytes than other isolates. Our studies suggest that co-receptor use by HIV primary isolates is an important, but not the sole determinant of HIV pathogenesis in the thymus. Experience with these *in vitro* models will be exploited to examine the capacity of virus recovered from HIV infected adolescents to disrupt T cell differentiation in thymic tissue.

Distribution of T cell receptor Vb types in HIV infected individuals

Cellular immune responses to HIV are insufficient to clear all infected cells. In part, this may be result from the selection of viral escape mutants. Theoretically, a cytotoxic T cell (CTL) response that targeted a given epitope with several distinct T cell receptor variants would be expected to more effectively control HIV infection. To evaluate the breadth of the response of HIV specific CTL responses, the Jamieson laboratory has mapped the TCRVb usage of T-cells responsing to given epitopes using tetramer staining [86b] in which labeled synthetic peptides that mimic the structure of MHC I molecules are used to present synthetic peptides to the TCR of CD8 cells.

*As shown in Figure 7, CD8+ T-cells that bound a HLA-A*0201 tetramer presenting the gag derived, p17 epitope known as SL9 [86c] (SLYNTVATL) were present in three HIV infected men following seroconversion. Gating on tetramer positive cells, monoclonal antibodies were used to determine the TCR Vb types of the CTL that recognized SL9. A TCRVb antibody panel that includes mabs for Vb types 1,2,5.1,7.1,8,8,11,12,13.1,13.6,14,17,18,20,21.3,22, and 23 was purchased from Coulter/Immunotech, (Westbrook, Maine). Vb 3, Vb 5.2 /5.3 mabs were purchased from Pharmingen (San Diego, CA), and Vb6.7 was purchased from Endogen (Woburn, Mass). Vb13.2 was donated P. Marrack and was FITC conjugated by Sierra Lab Logics (Gilroy,CA). The proportion of each Vb was determined relative to CD8+ gag-tetramer positive lymphocytes. Note that the TCR Vb types that bind the tetramer vary between individuals ranging from 1 to 4. Studying a small group of selected subjects, we plan to determine and compare the breadth of the CTL response to SL9 in adolescents with HIV infection acquired perinatally or by adult behavior, as a parameter of the impact of HIV infection on immunological ontogeny in the PI-A.*

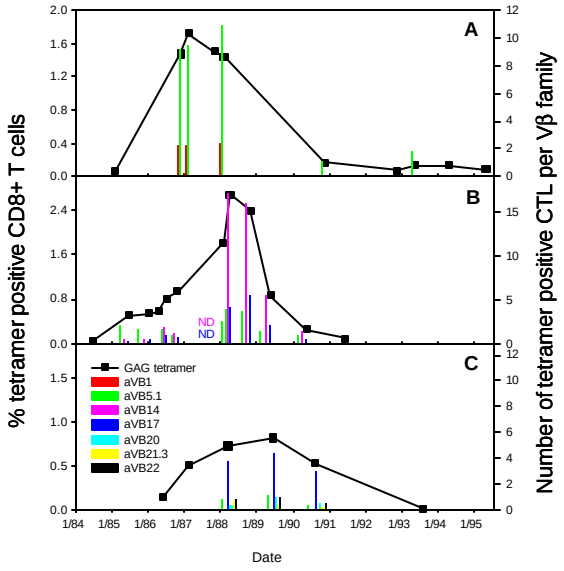


Figure 7. Vβ distribution of CD8 T cells responding to a Gag specific tetramer. Frozen CTL from three subjects (A-C) enrolled in the MACS cohort were stained with a tetramer representing the HLA A201 restricted Gag SL9 epitope (SLYNTVATL). Gating on the SL9-tetramer positive cells, specific antibodies were used to count Vβ cells of the types indicated by colored bar. **Left axis**: Frequency of SL9-tetramer positive cells among CD8 (Squares). **Right axis**: Number of tetramer-positive CTL. Each color bar indicates the number of CTL of a particular Vβ type.

D. Research design and methods.

We hypothesize that prolonged exposure to HIV replication in childhood and adolescence has an impact on thymopoiesis *that cannot be fully reversed by highly active antiretroviral therapy*. If so, *even after prolonged periods with undetectable plasma HIV RNA,* perinatally infected adolescents would be predicted to have small, atrophic thymuses, diminution of the naïve T cell subsets, decreased parameters of T cell production, and alterations in other parameters of thymopoiesis. As a corollary, the cumulative effects on thymopoiesis may have disrupted the development of cellular immune responses to HIV *(and other microbes)*, and narrowed the peripheral blood T cell repertoire. Conversely, the persistence of relatively preserved indices of thymopoiesis and T cell function in a perinatally infected individual may indicate that

the their virus populations have acquired attenuating mutations, perhaps in response to the selective pressure of antiretroviral therapy.

Recruitment of subjects

To test these experimental predictions, we will perform comprehensive and coordinated studies of parameters of thymopoiesis in *selected* perinatally infected adolescents and adults (PI-A) and two age-matched *(13 to 21 years of age)* control groups: seronegative individuals (SN-A), and adolescents and young adults who have acquired HIV infection by adult behavior (AB-A), e.g. sexual contact or the use of illicit drugs. Adolescents will be excluded if they have evidence of chronic Hepatitis B or C infection. Subjects will be enrolled after obtaining consent obtained under the guidelines of the University of California, Los Angeles, and Childrens Hospital Los Angeles (CHLA) Institutional Review Boards. We plan to enroll perinatally infected subjects 13 to 21 years of age of either gender, and irrespective of race and ethnicity. Younger children will be excluded, as this study will focus on HIV pathogenesis in puberty and post-pubertal adolescents. *Subjects who acquired HIV infection via transfusion in the immediate newborn period would be allowed to enroll in the PI-A group.*

In the first submission, we proposed a comparison of parameters of thymopoiesis in PI-A, AB-A, and SN-A, based on our estimates that we were likely to be able to recruit up to 30 PI-A. Its reviewers understandably highlighted the importance of controlling for the type of antiretroviral therapy, and consideration of an intra-group analysis of PI-A. In response, we surveyed the characteristics of patients being followed at our three Los Angeles clinics (Table 3), and found that a much larger number of PI-A are being followed than we had suspected, including 22 perinatally infected subjects who have had undetectable HIV RNA levels for more than one year while receiving HAART. FourPI-A were known to have acquired HIV by perinatal transfusion.

Table 3: HIV infected adolescents 13 - 23 years of age	**PI-A**	**AB-A**
Not on antiretroviral therapy (ART)	7	35
On ART, HIV RNA <400 copies /ml for one or more years	22	17
On ART, HIV RNA 400 to 10,000 copies /ml.	16	3
On ART, HIV RNA > 10,000 copies /ml.	15	1
Total	**60**	**55**

These numbers will increase in the years ahead, as other perinatally-infected children age, and adolescents infected by adult behavior are identified, enter care, and begin therapy. With the ability to work with groups of approximately twenty subjects, we will therefore focus primarily on two types of analysis involving perinatally infected subjects:

1. *A comparison involving PI-A and AB-A who have had successful suppression of HIV plasma HIV RNA by HAART, and SN-A of comparable age.*
2. *A comparison of the three groups of PI-A on HAART, stratified by their viral load levels over the previous year (HIV RNA undetectable, 400-10,000 copies/ml, or >10,000 copies/ml).*

Overall plan of evaluation (see Table 4).

Table 4: Evaluations planned

		Adult behavior	Perinatally infected			
	Uninfected	HAART	HAART			Untreated
		VI<400	VL<400	VL 400-10,000	VL >10,000	
Parameters of thymopoiesis						
Lymphocyte subsets	X	X	X	X	X	X
TREC assays	X	X	X	X	X	X
Thymus volume by CT	X	X	X	X		
In vivo labelling	15	10	10	10		
Virological analysis						
Detection of CCR5 Δ32		X	X	X	X	X
nef gene sequencing		X	X	X	X	X
pol gene sequence analysis			Virus	Virus/plasma	Virus	
Isolation of HIV/thymic organ culture		5	5	5		
Immunological studies						
Elispot with HIV library		X	X			
Elispot with CEF panel	X	X	X	X		
Lymphocyte proliferation assays	X	X	X	X		
Vβ repertoire-HIV		5	5			
Vβ repertoire-influenza	5	5	5			

Upon enrollment, we will obtain a blood sample for evaluation of T cell subsets and quantitation of recent thymic emigrants by TREC assay from all PI-A.

In selected subjects, the breadth of cytotoxic T cell responses to HIV and other viral pathogens will be evaluated by Elispot, and the breadth of the T cell repertoire response in certain HIV and influenza specific CTL responses. We will determine if any of the HIV infected subjects carries the D32 mutation of CCR5, or is infected with nef defective virus. The volume of the thymus will be assessed using volumetric computed tomography, excluding untreated PI-A, and those with higher HIV RNA levels (>10,000 copies/ml). If after the twelve-month visit substantial changes in measures of thymopoiesis are seen, a second CT scan evaluation of the thymus will be performed at the 18-month visit. For selected subjects (see Aim 2) HIV will be isolated in-culture by standard or ultrasensitive methods. The replication properties of these virus isolates will be determined in lymphocyte, thymocyte, and human thymus organ culture. We will perform nucleotide sequence analysis of plasma HIV RNA from PI-A with discordant treatment responses (CD4+T cell counts >200 cells/ml and ≥15% of total, ***and*** *plasma HIV RNA 400-10,000 over the previous year),* and also sequence the virus isolates to determine the impact of resistance-associated mutations on *in vitro* replication, T lymphocyte values, and parameters of thymopoiesis. Because replication in thymic tissue has been correlated with CXCR4 utilization, virus tropism of the virus isolates will also be examined using CD4+ Hos cell lines that express individual chemokine receptors, and in blocking experiments in thymocyte culture.

Three additional visits are planned for all subjects, at 6, 12, and 18 months into the period of follow-up (See table 5). These will allow for a long period of follow-up during puberty, for younger PI-A and those with delayed onset of puberty. This period will also provide a sufficient interval to reveal changes in T cell subsets, and, possibly in parameters of thymopoiesis. At each visit, physical (Tanner Staging) and, *if development of secondary sexual characteristics is incomplete*, hormonal assessments of puberty will be performed so that any changes in thymic function can be correlated with progression of puberty. At the six-month visit, we plan to initiate *in vivo* labeling experiments with *15* clinically stable SN-A, *10* PI-A, and *10* AB-A using glucose containing the stable isotope deuterium. This will require an overnight stay in the Clinical Research Center, and two additional visits two and four weeks later for blood sampling for subjects enrolled in this sub-study. Prorated payment of incentive fees will be offered to encourage compliance with this experimental regimen.

Table 5. Schedule of Visits	0	6	12	18	24	30
T cell subsets	All	All	All	All	PRN	PRN
TREC	All	All	All	All	PRN	PRN
Virus isolation	Selected PI-A/AB-A			Latent infection frequency++		
CT Scan	All			PRN	PRN	PRN
CTL Mapping		Selected*		Selected*		
Resistance sequencing#	Selected PI-A.					
Nef	PI-A/AB-A					
CCR5	All					
Metabolic labeling^		15 SN-A, 10 PI-A, 10 AB-A,				
Vb profile	Selected**		Selected**			
Hormone levels	Pubertal	Pubertal	Pubertal	Pubertal	Pubertal	Pubertal

PRN = if needed for extended follow-up of subjects who have had a change in therapy.
All = all PI-A, AB-A, SN-A.
* Samples from 5 control subjects will be used to determine background responses to peptide library. PI-A and AB-A with HIV RNA < 400 copies/ml during at least one year of HAART will be studied.
++ Frequency of latently infected cells will be examined in selected AB-A and PI-A (See text).
** Cryopreserved speciments from 5 selected PI-A, AB-A, and SN-A from these timepoints will be used.
# Sequencing of plasma HIV RNA in PIA with "discordant" response profile.
^ Metabolic labeling requires two blood draws, at 2 and 4 weeks after the initial 6 month visit.
Pubertal: Only for subjects not at Tanner stage 4 or 5 at initial visit.

It is anticipated that most perinatally infected subjects will maintain a given antiretroviral regimen during this time frame. *If the therapy of a PI-A changes significantly (e.g. an entirely new antiretroviral regimen is initiated), two additional visits will be added to allow us to examine prospectively the impact of this change.* Follow-up of adolescents may therefore continue for a total of 30 months. Enrollment is expected to be completed in year 2, and all study visits completed by the end of year 4. Table 5 lists a schedule of these visits. This

schedule is designed to allow all planned studies, without exceeding institutional guidelines for phlebotomy (1 ml/kg/month). The remaining immunological and virological laboratory studies and data analysis will be completed in year 5.

Division of Labor

Dr. Krogstad will have primary responsibility for the oversight of all studies, enrollment of subjects at UCLA, supervision of sample processing, immunophenotyping, sequencing of *nef* and TREC analysis. He will also be responsible for the management of subjects having *in vivo* labeling studies in the UCLA CRC. Drs. Marvin Belzer and Joseph Church will be responsible for the enrollment of clinical management of subjects at CHLA. Drs. Yvonne Bryson at UCLA will be responsible for clinical virology (virus isolation and genotypic resistance testing). Dr. Christel Uittenbogaart will be responsible for thymic organ culture studies. Drs. Otto Yang and Beth Jamieson will perform in depth immunological studies to assess the diversity of the peripheral T cell population and cellular immune reponses. Dr. Ines Boechat will perform the tomographic analysis of thymus volume. Analysis of samples from metabolic labeling experiments will be performed by Dr. Marc Hellerstein, who has pioneered these techniques in studies of HIV infected adults. Dr. Christina Ramirez-Kitchen of the UCLA Biostatistics Department will maintain and be responsible for analysis of the complex dataset resulting from this study.

Clinical Sample Processing

Blood specimens from subjects enrolled at Children's Hospital Los Angeles (CHLA) will be held at room temperature and delivered by courier to UCLA for flow cytometry and sample preparation within 24 hours. Plasma will be separated and stored at $^{-}70^{o}$C, and PBMC will undergo cryopreservation by standard methods. The clinical laboratories of UCLA and CHLA will perform the HIV plasma RNA assays. *The CHLA Research Immunology/Bone Marrow Transplantation Laboratory will perform lymphocyte proliferation assays with tetanus and Candida antigens.*

Statistical Considerations. We will use several methods to examine thymic function and output as it relates to HIV disease and antiretroviral therapy. Measures used to evaluate thymopoiesis include volumetric measurement of the thymus via computed tomography, T-cell subset panels, quantitation of TREC in total PBMC, purified CD4+, CD8+ and naïve CD4+T-cells, and metabolic labeling of T cell populations. *In our preliminary studies using PBMC samples from 14 to 19 year old seronegative subjects (Figure 2), we found a mean log_{10} transformed value of 4.58 copies/mg PBMC DNA (Standard deviation 0.227). Similar values were found for AB-A who had undetectable HIV RNA while on HAART (n = 3): (Mean= 4.85, SD=0.220). Only two PI-A with long-term suppression of HIV RNA have been examined so far, including the subject described in association with Figure 3 (Preliminary studies). These two subjects had surprisingly high TREC values. Assuming a similar distribution of values among PI-A subjects with HIV RNA under 400 copies/ml for long periods, we will have 80 percent power at an α level of 0.05 to detect a log_{10} transformed difference in TREC values of 0.20 with a sample size of 20 per group, using a two-sided test. Using the variance in values from metabolic labeling studies from adults* [45,60a], *we project that a 30% difference in calculated T cell turnover rates could be detected using groups of 15 seronegative subjects, and ten HIV infected subjects, with 80% power and an α level of 0.05 using a two–sided T-test.*

We will examine the effectiveness of thymic output by several measures: examination of Vβ distributions in selected subjects, lymphoproliferative responses to recall antigens, and quantitation of cellular immune responses to HIV by using ELISpot to quantify responses to a library of peptides. *We will use Kolmogorov-Smirnov testing to compare differences in Vb repertoire between subjects. (Kolmogorov-Smirnov is a powerful non-parametric method to compare differences between distributions).* The relationship between different quantitative variables will be measured by Spearman's rank correlation coefficient. We will also examine the relationship via Mixed-models repeated measures regression. *We will then quantify the qualitative variables by several methods, such as categorization and the use of dummy variables.* Once all the variables have been quantified, we will relate them by Spearman's rank correlation coefficient and Mixed-Models repeated measures regressions. Differences between uninfected and infected groups will be examined by Wilcoxon's Rank Sum Test and repeated measures t-tests.

Aim 1. To compare quantitative parameters of thymopoiesis in perinatally infected adolescents (PI-A), seronegative adolescents/young adults (SN-A) and adolescents with HIV infection acquired via recent adult behaviors (AB-A).

Hypothesis: In this aim, we will address the hypothesis that long-term survival after perinatal HIV infection *impaired thymopoiesis*, reducing thymic mass, T cell production, and the size of the peripheral naive T cell pool.

Rationale: The cumulative effect of lifelong HIV infection has not been examined in infected adolescents and young adults, but HIV infection has been associated with a reduction in thymic mass and disruption of thymic architecture in adults and children. Decreased thymic output (indicated by measurements of T cell receptor excision circles) has been seen in HIV infected children and adults, suggesting a direct impact on the thymus. Small radiographic studies have demonstrated a reduction in the size of the thymus and rare cases of cystic dysplasia in younger children, but adolescents with life-long HIV infection have not been specifically examined. Only limited studies have examined the correlation between these parameters of thymopoiesis, and the impact of progression of puberty on thymic function of HIV infected children has not been evaluated.

Strategy: Measurement of T cell receptor excision circles (TREC), flow cytometric measurements of lymphocyte subsets, volumetric radiographic imaging of the thymus, and *in vivo* metabolic labeling of T cells will be used to provide a comprehensive assessment of thymic activity and the character and turnover of peripheral T cell subsets. These measures will be correlated with clinical and laboratory parameters of pubertal development.

Flow cytometry and measurement of T cell receptor excision circles (TREC).

We will immunophenotype peripheral blood T cell subsets by whole blood staining of specimens with FITC, APC, and PE labeled antibodies using 50µl blood for each combination. The antibody combinations planned include CD45/CD14 (to aid in identifying the lymphocyte population), CD3/ CD4/ CD8, and CD4/CD45RA/CD62L (naïve CD4 T cells), and CD8/CD45RA/CD62L,CD103 (naïve CD8 T cells). *An additional tube will be run to determine the number of Ki67 positive CD4 and CD8 positive T cells, as an index of cell proliferation (CD3/ CD4/ CD8, Ki67).*

TREC assays will be performed on purified lymphocyte subsets, as well as on total PBMC. In most studies published to date, TREC in total PBMC have been evaluated. In our preliminary studies, we found PBMC TREC values to be very stable in uninfected adolescents, and in a small number of cases examined, there was a good correlation between TREC values in CD4+ and CD8+ T cells, and with PBMC (data not shown; see also reference). Still, there may be significant differences in TREC values in CD4+ and CD8+ cells of HIV infected subjects, so these subsets of will be purified from cryopreserved PBMC *using negative selection (Stemcell Technologies)*, the purity of these cells will be checked by flow cytometry with CD45/CD14 and CD3/ CD4/CD8 antibody combinations. Using real time PCR, TREC will be quantified in total PBMC (for comparison to other studies) and in the purified CD4 and CD8. DNA from CEM or Jurkat T cells lysed in concert with experimental samples is used as template in negative control reactions.

Volumetric computed tomography of the chest

Non-contrast CT will be performed without sedation at UCLA or CHLA by a standard protocol, scanning from the thoracic inlet to mid-chest, with 3 mm collimation. The volume of the thymus will be determined from spiral CT data in the Quantitative Image Analysis Lab (UCLA Department of Radiology). Custom software is used that applies a parametric model of anatomy to guide image segmentation to identify the boundaries of the thymus in three-dimensions, and then sums the volumes of the included voxels [73-75] . Following the scheme used by McCune et al [44] , the degree of replacement of thymic tissue with fat will be estimated by Dr. Boechat, who will be blinded to clinical characteristics of the patients.

In vivo metabolic labeling.

We will use in vivo stable isotope labeling to directly measure T cell turnover and estimate turnover in subjects from each of the three study groups: PI-A (n=10), AB-A (n=10), and SN-A (n=15), using stable methods previously described by Hellerstein et al and McCune et al [45, 59] . In brief, $^{2}H_{2}$ glucose is administered intravenously and incorporated through the de novo nucleotide synthesis pathway into the DNA of dividing cells. After a fixed labeling period, a period is allowed for distribution of cells, and

peripheral blood samples are obtained. Mulitparameter fluorescence activated cell sorting is used to purify lymphocyte subsets, and the DNA from these cells is extracted, and subjected to enzymatic hydrolysis. The rate of constant of replacement of unlabeled DNA strands by labeled DNA is calculated. From this parameter (the fractional replacement rate = k), the absolute production rate and half-life of the cells is calculated.

We plan to administer 100 to 200 grams (2 g/kg) of 6-6-$^2H_2$ glucose by constant infusion over 24 hours in the Clinical Research Center at UCLA Medical Center. Blood draws will be obtained at 12-hour intervals to determine the degree of enrichment of the peripheral glucose pool with the labeled glucose. After 4 to 7 days, and 8 to 14 days, 30 to 50 ml blood draws will be obtained and T cell subsets will be purified by preparative flow cytometry in the UCLA CFAR Flow Cytometry Facility. (The allowable limit would be a total blood draw of 1 ml/kg/month). The deuterium enrichment of the DNA of CD4+ and CD8+ T cells, and the CD4+CD45RA+CD62L+ populations will be measured in the laboratory of Marc Hellerstein, to yield estimates of the turnover rates and the daily production rates of these cell populations.

We plan to examine these parameters in adolescents that have peripheral blood CD4 T cell counts of at least 150 cells/μL; in the adult cohorts previously described[45,60a], DNA labeling could not be detected in those with cell counts much lower than this mark. Our preliminary data suggest that the majority of PI-A have T cell counts at least this high. However, the daily production rate of naïve CD4+ T cells over a 24 hour of labeling may be too low to be detected using a 24 hour period of 6-6-$^2H_2$ glucose infusion. If we are unable to quantify the labeling of DNA in CD4+CD45RA+CD62L+ cells with the one-day labeling period, label can be administered for longer periods in the form of deuterated water given to the subjects. With this approach, subjects will stay *8 to 12 hours, or overnight,* in the Clinical Research Center to receive oral aliquots of 70% D_20 (Cambridge Chemical) to begin to increase their body water content of D_20. (This step is necessary because rapid enrichment of the body water may produce transient vertigo in some subjects. This phenomenon resolves within the first day, as equilibration occurs) (Marc Hellerstein; unpublished observations.). They will be given pre-measured aliquots of D_20 in plastic vials to drink 2 to 3 times per week to replace deuterium lost by body water turnover. The labeling will be continued for four weeks, with blood samples drawn on days 14 and 28. As described above, we then calculate the turnover and production rates of the total CD4 and CD8 T cell populations, and the naïve CD4+ T cell subset. A similar approach has been used successfully to measure naïve and memory/effector phenotype T cell kinetics with D_20 in adult humans [60] , as well as in rhesus macaques (see preliminary data).

Tanner Staging and hormonal parameters of puberty.

Clinical assessment of puberty will be performed at the initial visit, using the 5 point Tanner scale of genital and breast development. If Tanner 5 (fully mature) secondary sexual characteristics are not present, these measures will be repeated at subsequent visits to track pubertal changes.
In addition, serum levels of testosterone (for males), estradiol for females), and insulin like growth factor (IGF-1), will be obtained as an objective measure of pubertal hormonal changes.

Interpretation of Results

We plan to examine the statistical correlation of several measures of thymic mass and activity *in HIV infected and seronegative adolescents*. The most straightforward of these measures is the use of flow-cytometry to quantify CD4+ CD45RA+CD62L+ T cells, generally accepted to identify naïve CD4+ T cells. This combination of markers is found on CD4+ cells that have a relatively long half-life, and thus cannot be used to identify recent thymic emigrants. TREC values appear to provide an analytically consistent estimates of thymic output, provided that naïve T cell proliferation levels remain low [55.25a]. *In this study, Ki67 will be used to provide a parameter of CD4 and CD8 T cell proliferation, as described by others [29b].* Naïve CD4+ T cells are thought to be maintained without proliferation, and we have therefore chosen to focus on TREC values in this subset. However, we will also quantify the CD103 subset of naïve CD8+ T cells, since this marker appears to identify recent thymic emigrants. Although we think it is not feasible to plan on performing metabolic labeling studies in all subjects enrolled in this study, we plan to study a sufficient number of carefully matched HIV infected and uninfected subjects to allow us determine the effect of T cell turnover on TREC values in the CD4 and CD8 T cell subsets, and to use naïve CD4 T cell production as a parameter of total thymic output. *We will also have the opportunity to determine the utility of using Ki67 and TREC assays in concert to evaluate thymic activity. In one recent report, [29b] there was an excellent correlation*

between Ki67 expression levels and T cell production rates in both CD4 and CD8 cells. If validated by comparison to results from metabolical labeling, Ki67 or TREC values could serve as a surrogate marker for thymic output.

In general, aging does not consistently produce a decrease in the volume of the thymus; HIV infection reduces the overall size of the thymus to below limits expected for age in many individuals, and thymic cysts have been reported in younger children. Radiographic estimates of thymic volume are clearly an imperfect measure of the amount of functional thymus present in an individual. Although fat replacement can be identified [43, 44] , tissue with the properties of normal thymus is not necessarily functional epithelial tissue that can participate in thymopoiesis; invasion of perivascular space by memory T cells may mask involution of thymic epithelial space [30] . Our rationale for including radiographic imaging in this study is that a marked reduction in thymic volume despite HAART, may reveal an acceleration of the natural rate of involution of the thymus. This is of interest, as we hypothesize that over time, those with a smaller amount of functional tissue (the most atrophic thymuses) may not be sufficient to maintain TREC values and naïve T cell populations.

We will follow TREC values for up to 18 months in patients with stable therapy. If a decrease in TREC values is seen in pubertal children, we will examine the correlation with changes in estradiol and testosterone concentrations. From studies, an increase in testosterone would be expected to correlate with a decrease in thymic output.

One focus of our analysis will be a comparison of parameters of thymopoiesis in PI-A to control populations of AB-A and SN-A. The study of age-matched SN-A will yield unprecedented normative data about thymopoiesis in adolescence, that may prove invaluable for further studies of the steadily increasing population of PI-A. By comparing only those AB-A and PI-A subjects who have had undetectable HIV RNA levels for more than a year on HAART, we will be able to minimize confounding by variation in therapy and viral load. Comparisons of T lymphocyte production rate, TREC assays, Naïve T cell numbers, and other parameters will allow us to determine if there are abnormalities in thymopoiesis that are not reversed by lengthy periods of HAART. By comparison of the AB-A group to the SN-A, we will also obtain an estimate of the effect of short term HIV infection on thymic volume, T cell production rate, and TREC values. These, too, are largely novel data (although the REACH cohort has provided important information about T cell subset abnormalities in AB-A)(70a).

We also plan to examine parameters of thymopoiesis in PI-A with "discordant" treatment responses, and compare them to those from SN-A, and the PI-A and AB-A with "undetectable"viral load measurements. Thus, we will determine if normalization of T cell parameters occurs in discordant treatment responses in adolescents, as has been reported by Deeks et al in a study of substantially older (mean age 40 years) adults (Appendix) . These data will undoubtedly help elucidate the nature of the "discordant treatment" state which occurs commonly in PI-A (See table 3).

Potential pitfalls and alternative approaches

Enrollment and retention of subjects: We had little difficulty in enrolling 14 adolescents with perinatal infection in an initial cross sectional study of TREC values, and anticipate little difficulty in enrolling these in the broader study we are proposing. *As noted above, 60 perinatally infected individuals 13 years of age and up are being followed in the CHLA and UCLA clinics.* Significant drop out of these patients is not expected, as they generally have remained associated with these institutions. With incentive payments, we hope to retain 20 uninfected control subjects and 20 youths with HIV infection acquired by adult behavior, as well.

The Risk Reduction program of Childrens Hospital Los Angeles has demonstrated its ability to accrue and retain HIV-positive and high risk adolescents ranging from age 12 years to 24 years through its excellent performance on the Adolescent Medicine HIV/AIDS Research Network (AMHARN). During Project REACH in this site has enrolled a total of 45 subjects, 13 to 19 years of age. Twenty-six of these subjects acquired HIV through either unprotected sex or injection drug use. The remaining 19 subjects were HIV- negative youth who are at very high risk of acquiring HIV due to their engagement in behaviors such as unprotected sex and injection drug use. Retention of HIV-infected and high-risk youth in the REACH study was extremely successful. Throughout the course of the study, only two control subjects were lost to follow-up, and no HIV-positive subjects were lost to follow-up.

In February, 1999, Dr. Belzer was awarded funds for a special "Adolescent Initiative" funded by the AIDS Clinical Trials Group (ACTG) to sites who demonstrated their ability to recruit and retain HIV-infected youth between the ages of 13 and 24 in clinical trials. The Childrens Hospital Los Angeles Adolescent Initiative site recruited nine HIV-infected youth into ACTG 381. Of the nine subjects enrolled, two have been withdrawn from the study, one relocated to Northern California and one is lost-to-follow up. Concerted efforts are made by the study

coordinators and Dr. Belzer to recruit and retain subjects into the research studies offered at Childrens Hospital Los Angeles. Through the multiple programs offered within the Division of Adolescent Medicine, clients learn of the various research opportunities and are connected to Dr. Belzer or one of the study coordinators. The numerous outreach efforts and existing liaisons contribute to the current success of the Risk Reduction Programs recruitment and retention of study participants. Patient compliance and continued participation in research studies can be directly contributed to the positive relationship that the staff develop with subjects, as well as tracking and maintaining contact with research participants. We have found that the key to retention is meeting the needs of our patients through a highly trained, multidisciplinary team who can provide support and linkages to community services.

Matching of PIA to control groups. *The PI-A examined thus far have generally been on several regimens of therapy, yet generally have detectable HIV RNA, but, as noted above, we have confirmed that a large number of PI-A with HIV RNA < 400 copies/ml are potential candidates for study. Most AB-A followed at our institutions are either on treatment with undetectable viremia, or not on treatment with lower levels of viremia and stable, higher levels of CD4+ T cells (in accord with current PHS guidelines [87] . The PI-A and AB-A subjects with prolonged periods with plasma HIV RNA <400 copies per ml have clearly demonstrated themselves to be adherent to treatment regimens. Consequently, we have the opportunity to compare parameters of thymopoiesis (and other immunological parameters in Aim 3) between adolescents who differ by having (or not having) had exposure to HIV throughout infancy and childhood, As noted above, we plan to compare parameters of thymopoieses between PI-A on HAART who have undetectable HIV RNA, and adolescents with higher levels. Data from age matched uninfected youths and AB-A with undetectable HIV RNA will offer additional comparisons.*

In vivo metabolic labeling . Adherence is more assured with the deuterated glucose labeling method, using prorated incentive payments to encourage subjects to return for the blood sampling for analysis, but it may not be sensitive enough to measure naïve T cell production rates in adolescents. Initial studies with deuteurated glucose will reveal this. D_2O is a more sensitive for slow-turnover cells, but will require extra efforts to ensure that the adolescents maintain the replacement regimen (small amounts of D_2O given 3 to 7 times per week) and return for blood draws. Frequent telephone follow-up by clinic physicians and study nurses will be used to remind subjects to drink the deuterated water. Fortunately, the turnover of body water is slow, which limits the impact of occasionally missed "doses" on the deuterium content of body water.

Tanner scale assessment. Assessment of pubertal development by Tanner staging is somewhat subjective, but this is generally problematic only when attempting to track progression from onset of puberty (e.g. initial formation of dark pubic hair to its accumulation) to more mature stages. We are concerned in this study with documenting that that puberty has progressed to a point where high levels of Igf-1 and sex steroids are present, as these have been shown to modulate thymic involution in murine models. Measurement of IGF-1, testosterone, and estradiol will provide confirmation that this is the case.
CT Imaging. As noted above, several processes can lead to enlargement of the lymphoid tissue of the thymus. Follicular hyperplasia may enlarge the thymus in some subjects. In others, compensatory hypertrophy could be present, although it is unclear if thymopoiesis is controlled in a homeostatic fashion. (Limited data indicate that IL7 levels, and perhaps other factors, increase with CD4 T cell depletion [88]). The period of observation planned may be too brief to detect decompensation and reduction of thymic volume, even if perinatally-infected adolescents are experiencing accelerated involution of the thymus. Nonetheless, we may be able to establish a meaningful correlation between the amount of thymic tissue present, and parameters of thymopoiesis and naïve T cell subsets.

Aim 2. To evaluate the impact of viral factors on thymopoiesis of HIV infected adolescents.

Hypotheses: *HIV infection of cells in the thymus may be common in perinatally infected adolescents, resulting in thymic injury and accelerated involution. However, mutations in the viral nef gene, pol gene mutations selected by antiretroviral therapy, and other virus properties may limit replication of HIV in thymic tissue, reducing the impact of perinatal infection on thymopoiesis.*

Rationale: *Host-virus interactions, and properties of an individual's viral quasispecies are likely to contribute to the wide variation in the rates of progression of disease of perinatally infected adolescents, and in their responses to HAART.* For example, deletions and mutations in the viral *nef* gene and the host CCR5 gene have been associated with long-term survival in both children and adults, *and must therefore be considered in any study of long-term survivors of HIV infection. In contrast, CXCR4 co-receptor use and in vitro syncitium-induction have been linked to disease progression.* However, recent studies indicate that chemokine receptor usage is only a partial determinant of the ability of HIV to replicate in thymocytes, and that drug resistant HIV variants demonstrate impaired replication in thymic organ cultures. Latent infection of naïve CD4 T cells has been detected in humans [39] and SCID-HU mice [38] suggesting infection of thymocytes occurs prior to emigration of naïve T cells from the thymus.

Strategy: *HIV will be isolated from selected PI-A and AB-A who have had undetectable HIV RNA levels with HAART, and from PI-A with discordant treatment responses to HAART (good immunological response, with persistently detectable HIV RNA).* The replication of these isolates will be examined in PBMC, thymocyte, and thymic organ cultures. We will also determine if drug resistance or *nef* gene mutations are present that could be modulating the virulence of the virus variants present in these adolescents. The frequency of latently infected naïve CD4 T cells will be examined in PI-A and AB-A subjects *with undetectable HIV RNA.*

Patient virus stock preparation

For PI-A with discordant treatment responses, virus isolation will be attempted from plasma samples, using allogeneic activated CD4+T cells (as previously described [89]). Briefly, allogeneic CD4 cells from three normal donors are individually purified by capture in CD4 mAb-coated tissue culture flasks (Applied Immunosciences, Santa Clara, CA) and activated by stimulation with antibody to CD3 and with rIL-2 (5000U/ml) for 5 days. Cells from three donors are combined, cryopreserved in liquid nitrogen, and then thawed and cultured in medium with IL-2 for 2 –3 days before infection. Plasma is added to these cells, and at weekly intervals ELISA for viral p24 antigen is performed. The same CD4 pool used for isolating virus from plasma (passage 1) will be subsequently used to expand virus stocks, if needed (passage 2). If plasma cultures are negative after two weeks, we will obtain virus isolates with a standard PBMC-HIV co-culture consensus protocol employed by the National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group. Viruses recovered from the first co-culture will be expanded by a single passage in the pooled CD4+ cells. The R5-tropic virus HIV-1_{JR-CSF} (JR-CSF) and the X4-tropic virus HIV-1_{NL4-3} (NL4-3), will be used as controls for the studies planned, and will be prepared in similar cultures. *In addition, we will use two molecularly cloned derivatives of NL4-3: 210WT and 210P. 210WT contains the protease domain of an HIV infected adult, cloned from a clinical sample obtained prior to therapy. 210P is a related clone that contains mutations in protease gene, found after patient 210 was treated with HAART [44a]. Stoddart et al found that these mutations attenuate replication in thymus organ culture [44b]. With the permission of F. Clavel, in whose lab they were made, we have obtained the proviral plasmids for these viruses from Dr. Stoddart, and prepared virus stocks by transfection of 293T cells, as described [44b].*

In patients without detectable plasma HIV RNA, we will obtain virus isolates from purified CD4+ T cells, as recently described [90] . Briefly, monocytes are depleted using anti-CD14 antibody coated magnetic beads. CD4+T cells are then positively selected with monoclonal antibody coated magnetic beads, and activated by incubation with soluble anti-CD28 monoclonal antibody in a flask coated with immobilized CD3 monoclonal antibody. The activated CD4 T cells are then cultured with the stimulated CD4+T cells. Virus replication is identified by viral p24 antigen ELISA of culture supernatants at approximately weekly intervals until a maximum of 42 days. Cultures are refed weekly with fresh, activated CD4 T cells from seronegative donors. In each case, if the patient is currently receiving a protease inhibitor, that agent will be added to the culture medium. The titer of the virus stocks will be determined by limiting dilution assays in PHA stimulated PBMC in triplicate wells. Wells in which the p24 antigen concentration is greater than 30 pg/ml after 7 days are scored positive, and the virus titer is calculated by the Spearman-Karber method.

Detection of latent infection in naïve CD4+ T cells.

In approximately 10 PI-A and AB-A, CD4+CD45RA+ T cells will be purified by fluorescence gated cell sorting (FACS) at the 12-month visit from freshly drawn PBMC. Subjects will be selected for this study whose naïve (CD45RA+CD62L+) CD4+counts have been at least 200 cells/μL blood in their previous 2

study visits, so that at least 1 million naïve CD4+ cells can be recovered by FACS. The purified naïve CD4+ T cells will be placed in culture for 48 hours to allow latent viral genomes to decay [91, 92] , then activated with CD3/CD28 co-stimulation. They will then be placed in limiting dilution culture assay with pooled allogeneic CD4 T cells, prepared as described above. The frequency of latently infected naïve CD4+ cells (IUPM=infectious units/million cells) will be calculated by the maximum likelihood method [93] .

Detection of *nef* and CCR5 gene mutations.

We will perform full length sequencing of the *nef* sequences to look for deletions or point mutations. DNA prepared from PBMC for TREC assay (or if unsuccessful, from PBMC with a Puregene DNA Isolation Kit, Gentra) will be serially diluted and trial amplifications performed with single copy sensitive nested PCR using: outer primer pairs 5'-AGAGCTATTCGCCACATACC-3' and 5'-TAGTTAGCCAGAGAGCTCCCA-3', inner primer pairs 5'-CTATAAGATGGGTGGCAAGTG-3' and 5'-TTATATGCAGCATCTGAGGGC-3'. These primers flank all of *nef*, and efficiently amplify sequences in NL4-3 and IIIB, and most patient sequences (data not shown). After establishing limiting dilution conditions, enough reactions to yield at least 10 amplifications will be performed for each sample, followed by sequencing with the same primers, and alignment with known *nef* sequences.

PCR will be used to determine if CCR5 Δ32 mutation is present in any of the HIV infected adolescents, using previously described primers to amplify a 184 base pair region that includes the site of the common deletion [68] . If evidence of a deletion is found, we will amplify the full size coding region of CCR5 gene, and confirm the presence of the deletion, as described by others [6] .

Nucleotide sequence analysis of resistance associated mutations.

For patients with detectable plasma HIV RNA, genotypic analysis of HIV RNA sequences will be performed using the Applied Biosystems ViroSeq HIV-1 Genotyping System (Applied Biosystems, Foster City CA). *(See Preliminary data, and manuscripts in appendix by Eshelman, Krogstad et al, 2001, and Cunningham, Ank et al, 2001).* In our recent pre-licensure collaborative pediatric study, PCR products suitable for sequencing were obtained for 192/196 samples, using plasma volumes ranged from 0.05 to 0.5 ml with viral loads ranged from 1,084 to 3,484,991 copies/ml. of the samples [83] . If the virus isolate used to study virus tropism and replication is derived from co-culture, we will also analyze sequence from the cell pellet DNA, to verify that the virus isolates contain viral sequences corresponding to the virus present in plasma. Identification of resistance-associated mutations will be based on the library maintained at the Los Alamos HIV Sequence Database (www.lanl.gov).

Examination of replication of patient-derived virus isolates in *In vitro* suspension cultures of thymocytes and thymic organ cultures.

We will examine the replication of patient virus isolates in thymocyte, lymphocyte, and thymic organ cultures. PBMC will be prepared from seronegative donors, stimulated with phytohemagglutinin for 3 days, then infected with virus isolates at a multiplicity of infection of 0.01. Virus replication will be monitored by p24 ELISA in samples collected on days 1,3,5, 7, and 10 post-infection. These cultures will allow us to initially examine the replication kinetics of each isolate, by comparing the rate of increase and maximum concentration of 24 antigen.

The replication and cytopathicity of patient derived virus isolates will be examined in both thymic organ culture and suspension cultures of freshly isolated thymocytes. The suspension cultures permit easy manipulation of exposure to various agents such as antiviral drugs. To evaluate the influence of thymic stroma and non-T cells on the replication of HIV in thymocytes, we will confirm the suspension culture data using organ cultures. HIV replication will be determined by following HIV p24 antigen in the supernatants (see below). *Cytopathicity will be evaluated by comparing changes in thymocyte subsets (using the antigens, CD1, CD4, CD8, CD3, CD27, CD69) in HIV infected with mock-infected thymocytes. Based on our preliminary data using primary pediatric isolates obtained at or close to birth, we expect to test each isolate in 5 different experiments to obtain statistically significant results. The variation of p24 levels of one isolate tested in different thymus specimens is about 3 fold. Therefore 5 cultures per isolate should give statistically significant results of differences in viral replication. The variation in depletion by one isolate of CD4 bearing thymocytes in different thymus specimens is about 10% and 5 cultures per isolate should give statistically significant results of differences in CD4 depletion. Based on data from Stoddart et al [44b], 3 to 30 fold differences in virus replication in thymic organ cultures may be seen on the basis of drug-resistance associated mutations.*

Normal pediatric thymuses from HIV-seronegative children are obtained in the course of corrective cardiac surgery. Single cell suspensions and nylon wool purification will be obtained as previously described [86] and the thymocytes will be cultured at 1-2 x 10^7 cells/ml in serum-free Iscove's modified Dulbecco's medium supplemented with delipidated BSA, and transferring, and IL-2 (20U/ml) and IL-4 (20ng/ml). These cytokines are chosen because they induce CCR5 expression, enhancing the detection of replication by R5 tropic virus.

For thymic organ cultures, fragments of thymus are excised from tissue specimens and cultured on floating rafts consisting of size 4 absorbable gelatin sponges covered with 0.8 micron nitrocellulose disks (MSI, Westboro, MA) in serum-free medium supplemented with IL-2 and IL-4.

To infect thymic organ cultures, thymus fragments are pooled in a 15 ml conical tube and incubated for 2 hours with virus or mock-infected supernatants in the presence of polybrene. After several gentle washes in medium without cytokines, 4 pieces of infected or mock infected organ tissue are returned to each duplicate Gelfoam raft, which had been pre-incubated for 1 hour in medium with different cytokines at 37 ^oC and 5% CO_2. The medium with cytokines was changed at day 1 post infection and every two days thereafter. Viral replication is assessed in the supernatant of individual wells by measuring viral p24 antigen, as described above, *and compared to reference strains, including NL4-3, JR-CSF, 210 WT and 210P (see above).*

Cellular depletion is measured by dual surface immunophenotyping of thymocytes with directly conjugated FITC and PE mAbs, as previously described. To exclude dead cells from the analysis, the thymocytes are incubated in a solution of 2µg/ml 7-aminoactinomycin D in PBS, washed in PBS and resuspended in 1% paraformaldehyde (PF) solution in PBS containing 4µg/ml of AD (9). For determining HIV expression in distinct thymocyte subsets, surface staining of thymocytes is performed, followed by fixation in PF, permeabilization in 0.2% Tween and cytoplasmic staining for HIV gag antigen with the KC57 mAb, as previously described [16, 85] .

To determine chemokine receptor utilization by the isolates, thymocytes will be infected and cultured as described above in the presence of 10 µg/ml polybrene. Control thymocytes are mock-infected in the presence of polybrene with supernatants from the same uninfected cells used to prepare the virus stocks. After infection the cells are washed and resuspended in serum-free medium in the presence of cytokines. To verify coreceptor use, thymocytes will be preincubated with the CXCR4-specific antagonist AMD3100 (1µg/10^7cells) or antibodies to CCR5 (2D7) (5µg/10^7 cells) and/or to CXCR4 (12G5) (10µg/10^7 cells) at 4^oC for two hours before infection. The antibodies and/or AMD3100 are present during infection and throughout the duration of the experiment. On day 1, and weekly thereafter, the medium will be removed and replaced with fresh medium containing cytokines and blocking antibody (or AMD3100). As internal controls for the specificity of the inhibitory effects, thymocytes are infected in parallel with the HIV_{NL4-3} and HIV_{JR-CSF} in the presence or absence of the antibodies to the coreceptors and/or AMD3100. Virus replication is assessed by ELISA of viral p24 antigen.

In the event that blocking experiments yield ambiguous results (no evidence of blocking by AMD3100 or anti-CCR5 mabs), chemokine receptor utilization will be determined using Human osteosarcoma cell lines (from N. Landau, obtained from the NIH AIDS Research and Reference Reagent Program) as previously described [90]. This might reveal usage of additional receptors, such as CCR8 [94]

Interpretation of Results

Although not yet detected in any of the PI-A examined to date, CCR5 mutations may be present in some perinatally infected adolescents, and would clearly be expected to retard the progression of disease. Similarly, gross deletions or significant mutations of *nef* would be expected to contribute to their survival. However, the PI-A examined to data appear to have suffered significant T cell loss from HIV infection, and many have had an AIDS defining illness. We therefore anticipate that wildtype *nef* and CCR5 will be normal in most individuals examined. Instead, it is likely that if defects associated with virus replication in thymocyte, lymphocyte and thymic organ are found they will be associated with protease or reverse transcriptase inhibitor resistance. The anticipated result, based on the recent report from Stoddart et al [44b], is that drug-resistant virus grows poorly in thymic organ tissue culture. Interestingly, some of the PI-A in our area are not currently receiving protease inhibitors. We will thus have the chance to determine if reverse transcriptase mutations are associated with poor replication in thymocytes and thymic organ cultures.

We plan to determine the frequency of latent infection of naïve CD4+ T cells from approximately *10* PI-A and *10* AB-A by limiting dilution culture of FACS purified cells. We will statistically examine the

correlation between the frequency of infection with TREC number, thymic volume, and the number of naïve CD8+ T cells. A correlation between these parameters would indicate a direct effect of infection of the thymus on thymopoiesis.

Potential pitfalls and alternative approaches

No intractable technical difficulties are expected in performing the studies we have outlined. Each of the assays described above is in routine use in the laboratories of Drs. Krogstad (genotyping of pol, CCR5 and *nef*), Bryson (virus isolation, including protocols to recover from patients with undetectable levels of HIV-1 RNA, and analysis of tropism), and Uittenbogaart (thymocyte and thymic organ culture). Specific technical concerns are addressed below.

Pol gene sequencing. We plan to detect resistance-associated mutations by bulk sequencing of the *pol* gene in our virus isolates and in plasma. Limiting dilution PCR amplification or initial cloning would allow us to estimate the frequency of mutant genomes in virus, plasma, and PBMC, but this information is not essential for this study. Bulk plasma sequencing should allow us to confirm the presence of mutations in part of the virus populations. Genotypic analysis remains an somewhat imperfect predictor of phenotypic resistance to antiretrovirals, but for this study we are more interested in examining the relationship of resistance-associated mutations of replication properties of virus in short term culture, rather than clinical outcomes.

Nef gene analysis. It may be difficult to determine the phenotype associated with *nef* mutations found by limiting dilution sequencing. Clearly, deletions, large frameshifts, and other clearly destructive mutations would be expected to have an impact on virus replication, and on the rate of progression of disease of PI-A or AB-A. Determining the phenotype of point mutants is more difficult to predict. Some mutants lead to poor replication in PBMC culture, yet have no affect on pathogenicity seen in the SCID-hu mouse system [96] . If interesting mutants are noted, these could be examined by taking advantage of the availability of the SCID-hu mouse core available at UCLA, (J. Zack, Director), using previously cloning strategies previously used in the Zack laboratory to examine *nef* mutants [96] . Additional funding would be sought for these animal studies, which go beyond the scope of this patient based proposal. We have chosen to target *nef* and *pol* gene mutations, and virus chemokine receptor use, as the likely viral properties that are playing a role in the survival of PI-A. Virus recovered from PI-A may replicate poorly in the human thymus organ culture system, yet have normal *nef*, and *pol* genes and chemokine receptor usage (CXCR4, CCR5, or both). Although beyond the scope of this proposal, these isolates could also be examined in SCID-hu mouse system, and substitutions of other regions of the genome into the backbone of HIV-$_{NL4-3}$ or HIV$_{JR-CSF}$ would allow us to identify other virus characteristics associated with decreased thymic replication.

Latent infection in naïve CD4+ T cells. We plan to determine if HIV infection is present in FACS purified naïve CD4 T cells, as evidence of infection of thymocytes in perinatally infected adolescents[39]. PI-A may have latently infected naïve CD4+ cells at very low frequency, making it technically difficult to exclude the possibility that the ultrasensitive culture system being used rescues virus from quiescent, recently infected cells containing labile, unintegrated viral DNA. We have considered the alternative approach of determining if any recent thymic emigrant **CD8+** T cells (CD8+CD45RA+CD62L+CD103+) harbor latent viral genomes, as this would offer assurance that infection was occurring during intrathymic T cell differentiation. In our experience to date, this population of cells is very small in HIV infected older adults (Jamieson et al, unpublished), but if immunotyping in Aim 1 shows us that the there is a sizable CD8+CD45RA+CD62L+CD103+ lymphocyte population in infected adolescents, we may be able to employ this experimental approach.

Aim 3. **To examine the breadth of cellular immune responses of perinatally infected adolescents.**

Hypothesis: Disruption of thymopoiesis due to longstanding childhood HIV infection may impair immunological development, compromising the generation of diverse cellular immune responses to HIV and other pathogens. *HAART may not be able to reverse this process, leading to a narrow ability to control acute and chronic infections.*

Rationale: HIV infected children often have atrophy and histological abnormalities of the thymus. Disruption of the thymic microarchitecture has also been demonstrated in tissue from HIV infected humans, and the human thymus/liver grafts of infected SCID-hu mice [30, 96-99] . Consequently, naïve T cells from long-term survivors of perinatal HIV infection may have limited T-cell receptor repertoire, and these individuals may have relatively limited CTL responses *to vaccines, HIV, and other infections*.

Strategy: Using a library of overlapping peptides to represent all HIV viral proteins, we will map and quantify CTL responses to HIV by Elispot assays in *PI-A and AB-A who have had HIV RNA<400 copies/ml for at least one year in response to HAART*. Data from these studies will be correlated with measures of thymopoiesis from Aim 1. *We will quantify cellular responses to Candida and tetanus antigens, and a pool of peptides representing influenza, cytomegalovirus, and Epstein-Barr Virus. In selected subjects, we will examine the Vb distribution of influenza and HIV specific CD8 T cells from PI-A and matched AB-A and SN-A, to determine if survival into adolescence after perinatal HIV infection is associated with a more narrow range of responses.*

Lymphoproliferative responses to Candida and Tetanus. Lymphoproliferation Assays (LPA) will be performed by the Research Immunology/Bone Marrow Transplantation Laboratory at CHLA by standard methods. Briefly, PBMC are obtained by Ficoll Hypaque gradient centrifugation of blood and cultured for 7 days in the presence of Tetanus toxoid (Axell) or Candida proteins (Bayer). Cells are pulsed with tritiated thymidine (1 µCi) 18 hours before the harvest. The results of proliferation assays are expressed as counts per minute (cpm) after subtracting the background (medium only) or as a stimulation index (SI, cpm of the stimulated sample/cpm of background). The mean values of triplicates are reported. A four-fold SI is considered significant, as described by others [46]..

Elispot Mapping cytotoxic T lymphocyte responses *to HIV and other viral pathogens.*

We will use Elispot assays to map thoroughly the T cell responses to HIV of *twenty PI-A and twenty AB-A with HIV RNA<400 copies/ml for at least one year in response to HAART.* Mapping and quantitation of CTL responses will be performed as described above in the Preliminary Studies, using a full set of Consensus B sequence peptides (15-mers overlapping by 11 amino acids) representing the entire HIV protein repertoire. Each of these subjects will be examined twice, using samples spaced one year apart. As controls, background responses to this library will be determined by examining responses from 5 to 10 seronegative age-matched control subjects.

To define HIV-1 specific CTL responses, patient PBMC will be screened initially against approximately 50 pools of 16 peptides or less, encompassing the entire library (Figure 5). To spare cells, the mapping will be performed initially on non-specifically expanded $CD8^+$ cells [100] . Positive pools will then be evaluated with 4 x 4 matrices as described above (Figure 6), with confirmation of individual peptides. Final quantitation will then be performed on unexpanded PBMC in triplicates, with quantitative analysis performed using an Elispot plate reader (Cellular Technology) available through the UCLA Immunology Core Laboratory. These studies will therefore define the breadth and magnitude of the HIV-1-specific CTL response.

In two additional wells, responses to common influenza, cytomegalovirus (CMV), and Epstein-Barr Virus epitopes (EBV) will be identified by Elispot, using a panel of peptides described by Currier et al [100a], *provided by the NIH AIDS Research and Reference Reagent Program (CEF Control Peptide Pool, Cat# 6747). We will also quantify responses to these peptides in 5 to 10 SN-A. These 23 peptides include 8-11mer peptides representing epitopes of influenza A (7 peptides), CMV (3 peptides), and EBV(13 peptides) presented by 11 class I HLA-A and HLA-B alleles found with cumulative frequency of >100% in white individuals. Data from these wells will provide an additional measure of immune function in PI-A, by comparison to data from the AB-A and SN-A.*

Examination of the Vβ repertoire of CTL Responses to highly conserved epitopes of HIV and Influenza.

We plan to characterize the breadth of the Vβ repertoire to HIV in 5 PI-A subjects and 5 carefully matched AB-A youths, using 5 SN-A as controls. Specifically, we will choose infected PI-A and AB-A with undetectable HIV RNA who have TREC values that are within 1 standard deviation of the mean of log-transformed values for uninfected adolescents (see statistical considerations). We will initially screen subjects during routine flow cytometry for the presence of HLA A2 using mabs available from One LambdaProducts. We will then identify PI-A and AB-A from among this group who show reactivity with a tetramer presenting the p17 Gag eptitope known as SL-9 [86c] (SLYNTVATL), *using methods described by others [86b]. Using a panel of antibodies to known Vb types, we will determine the Vb repertoire of these SL9-reactive CD8+ T cells, as shown in our Preliminary Studies involving*

samples from the MACS cohort (Figure 8). These studies will be performed using PBMC will be stored at the time of the initial visit, and we will obtain a second sample from each after an additional 12 months, so that each the profile from each subject can be evaluated at two points in time. This will allow us to determine the long-term stability of the Vb in the infected subjects. Although SL9-specific CTL decrease in number during HAART, Altfeld et al have shown that they can be easily detected after at least one year of HAART. [65].

*In a similar fashion we will examine the breadth of responses to influenza will be examined using a soluble HLA-A*0201/M1 58-66 tetramer described by others [86b]. Reactivity to the Influenza A virus M1 58-66 epitope predominately involves Vb 17, but other V b 17 types are also seen[100b]. As a recall response to an acute infection in the past, the number of reactive CTL may be too low to allow quantitation for comparison. We plan to examine samples obtained out between the months of April and November, to avoid stimulation by an acute infection, and initially increase the number of influenza-specific CTL by stimulating PBMC with a synthetic peptide representing the M1 58-66 epitope.*

The number of Vb families, and the number of cells of each Vb type found in response to the HIV or influenza epitopes will be quantified and the distributions compared to SN-A and AB-A using Kolmogorov-Smirnov method,(a powerful non-parametric method to compare differences between distributions).

Interpretation of Results

It has not previously been feasible to map thoroughly the CTL responses of sizable cohorts of HIV infected subjects. The development of Elispot has changed this, by making it possible to examine activation responses to large numbers peptides containing potential epitopes. In this study, the number of total peptides recognized (omitting those with overlapping amino acid sequences) and number of spot forming units per million cells will be used as parameters of the breadth and depth (respectively) of the immune response to HIV. *Responses to the panel of CEF peptides (CMV, EBV, Flu) have been methodically evaluated in both HIV positive and seronegative adults, demonstrating that this panel 1) is useful as a positive control panel for Elispot and 2) can reveal HIV-induced attenuation of responses to common recall antigens. Although we do not presently plan to map responses to individual peptides, these pooled peptides will provide an additional measure of CD8 T cell responsiveness. The data from age and treatment matched PI-A and AB-A will be compared by two-tailed T test. Based on our hypothesis that thymic injury has impaired thymopoiesis, and consequently narrowed the breadth of the peripheral T cell repertoire, we anticipate that PI-A will respond to a smaller number of peptides, and have lower numbers of responding CTL than AB-A matched for age and treatment status (we will compare only subjects with long term, stable responses on HAART). As a corollary, we expect that there will be a correlation between the number of viral epitopes recognized and the Vb diversity of the CD8 cells that respond to the SL9 and Influenza. We also expect that as a group, these PI-A will have more limited reponses to Candida and Tetanus in proliferation assays than the matched AB-A. It remains formally possible that at least a subset of PI-A will have broader responses to HIV and influenza than AB-A. These PIA would recognize a larger number of HIV and CEF peptides, and respond to the influenza and HIV epitopes with a broader diversity of Vb families than the AB-A individuals. Careful analysis of such subjects may reveal treatment factors or parameters of thymopoiesis that could prove useful in the prognostic evaluation of perinatally infected adolescents.*

We will also perform a more limited analysis of PI-A with discordant treatment responses, using only lymphoproliferative responses and the CEF Elispot panel. By comparison their LPA and CEF Elispot results with data from SN-A and the PI-A with undetectable HIV RNA, we will have a chance to determine if differences in functional immunological assays can be associated with the "discordant" phenotype in PI-A.

Potential pitfalls and alternative approaches The high sensitivity of the Elispot method should make it possible to perform the initial screening with the entire peptide library of CTL responses *in the selected HIV infected subjects.* We do not anticipate PBMC to be limiting for these studies, as mapping will be done on expanded CD8+ cells (expansion of a million PBMC yields approximately 10-20 million $CD8^+$ cells in our hands). Therefore, the number of cells available from 50-60 ml blood draws we plan should permit us to map all of the peptides to which an individual responds. These methods are all working in the laboratory of Dr. Yang, and we do not anticipate technical difficulties in defining these responses.

Meaningful comparison of Elispot data from the two groups of infected subjects necessitates that we carefully match these two groups for viral load, and the type of antiretroviral therapy being given [101] . The specific characteristics of therapy (i.e. antiretroviral drugs used) will remain a confounding factor that may be difficult to entirely control. *For this reason, we plan to emphasize the comparison of PI-A and AB-A who have had suppression of HIV viremia for a minimum of one year at the time of evaluation.*

E. Protection of Human subjects

Involvement of Human Subjects

We plan to recruit up to 60 adolescents and young adults from the Los Angeles area who have perinatal HIV infection. Subjects will be recruited who are 13- 23 years of age who have HIV infection that was acquired in infancy. An additional 20 control subjects will be recruited who have HIV infection that was acquired by other means. The subjects will all be recruited without regard to health status, except that we will exclude control-infected subjects who also have Hepatitis B or C infection as this may interfere with some assay measurements. Subjects will be enrolled without regard to ethnicity or gender. In addition, a group of 20 controlled subjects of similar age will be sought from adolescent medicine clinics, with the same exclusion criterion (no chronic Hepatitis B or C infection). Research material obtained from individually identifiable living subjects will include demographic parameters (age, gender, ethnicity), as well as clinical parameters directly related to their HIV infection (CD4 cell counts, current medications, and HIV RNA levels). Subjects will be recruited by posting handbills in clinics where HIV infected and uninfected adolescents seek healthcare at CHLA and UCLA Medical center. Consent will be obtained by nurse practitioners or physicians involved in the study, and information will be presented in a form of a language consent form. Informed consent will be obtained from each of their institutional review boards. All subjects will be asked to undergo a CT-Scan of the chest on one occasion. This is unlikely to present a significant risk, as the imaging technique that will be used as somewhat of a standard medical CT -Scan of the chest used for many pathological conditions, and will be performed without sedation or the injection of contrast materials. This is purely an observational study and no medications will be administered. Up to *60* subjects (up to *40* from the two groups of infected individuals, and up to *20* non-infected individuals) will be recruited by a similar procedure for metabolic labeling studies. These studies will involve a placement of an intravenous line and an infusion over 24 hours of a glucose containing solution. Minor risks associated of placement of an IV and having phlebotomy might come form this specific procedure. Risk of confidentiality will be minimized by having the principal investigator unaware of the identity of patients not being seen at UCLA Medical center, with anonymous identifiers being assigned by Childrens Hospital Los Angeles. In summary, this is an observational study that will consist of having a CT-Scan performed and 4-6 blood draws over a period of no longer than 30 months. Information gained from this study may not directly benefit subjects, but may provide vast information about the immunological consequences of long-term survival following birth

F. Vertebrate animals. Not applicable to these studies.

G. Literature cited.

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