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T Cell Immunity and HIV-1 Replication in Vertically-Infected Infants and Children: A Dissertation

Zachary Aaron Scott
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A Dissertation Presented

By

Zachary Aaron Scott

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

May 5, 2003

Program in Immunology/Virology
Experimental results presented in this thesis dissertation have appeared in the following publications:


T CELL IMMUNITY AND HIV-1 REPLICATION IN VERTICALLY-INFECTED INFANTS AND CHILDREN

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Zachary Aaron Scott

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Program in Immunology/Virology

May 5, 2003
DEDICATION

This thesis is dedicated to

My parents
Timothy and C. Jane Scott

My siblings
Joshua and Lindsey

And especially to
My best friend and beloved wife
Sarah McCombs Scott

Thank you for your support and love.
ACKNOWLEDGEMENTS

I thank my mentor Katherine Luzuriaga M.D. for a wonderful graduate experience as a member of her laboratory. As Katherine can attest, when I arrived at her doorstep 5 years ago, she had her work cut out for herself in transforming me into a young scientist. Through those years, Katherine was always there with helpful insight, encouragement, and enthusiasm for my research. I will always appreciate the time and effort Katherine has invested in me, and I will miss being a part of her team.

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ABSTRACT

Virus-specific cellular immune responses have been shown to be important in the control of viral replication in several animal and human virus models. Cells of both the CD8\(^+\) and CD4\(^+\) T cell lineages have been shown to play protective roles during viral infections by exerting effector functions that can kill infected host cells or inhibit the production and spread of infectious virions. The continued spread of HIV-1 infection throughout the world, as well as the lack of a prophylactic HIV-1 vaccine have generated much interest in HIV-specific cellular immune responses. Recent technical advances have yielded a tremendous increase in our understanding of HIV-1-specific immunity, as well as HIV-1 replication dynamics and host cell factors that shape the course of acute and chronic infection.

Unfortunately, due to small sample volumes and technological limitations, the study of HIV-1-specific T cell immunity in infants and children has been difficult. An improved understanding of the timing, specificity, and intensity of pediatric HIV-specific T cell responses would contribute to the development of a HIV-1 vaccine for use in regions of the developing world without access to antiretroviral therapeutics.

In the small number of published studies investigating pediatric HIV-specific immunity, T cell responses were uncommonly detected in infants. It remains unclear, however, whether the lack of HIV-specific T cells is an accurate reflection of the \textit{in vivo} immune state in vertically-infected infants, or rather is a consequence of reagents and assays ill-suited to the detection of low-level and/or diverse T cell responses in pediatric subjects.
In the present dissertation, several methodologies were used to investigate HIV-specific T cell responses in vertically-infected infants and children. HIV-specific CD8\(^+\) T cell responses were infrequently detected in a cohort of young infants, but are commonly detected in older infants and children. Interestingly, CMV-specific CD8\(^+\) T cell responses were detected in several young infants that lacked HIV-specific responses, suggesting a specific defect in the ability of some infants to generate HIV-specific CD8\(^+\) T cell responses. Further experiments characterizing detectable HIV-1-specific CD8\(^+\) T cell responses found that the HIV-1 accessory proteins may be important targets of the immune response during early vertical infection. The role of HLA class I genotype and viral sequence are also explored in a pair of vertically-infected twins with discordant CD8\(^+\) T cell responses. Finally, viral isolates from an infant with a marked shift in gag-specific epitope usage during infancy are analyzed for the presence of escape mutations.

Gag-specific CD4\(^+\) T cell responses were commonly detected in a large cohort of vertically-infected children. A linear relationship between HIV-1 replication and the presence and intensity of HIV-specific CD4\(^+\) T cell responses was found, but ongoing HIV-1 replication appeared to blunt CD4\(^+\) T cell proliferation.

The data presented in this dissertation describe pediatric T cell immune responses and how they relate to HIV-1 replication. This information may be useful to the design of a prophylactic or therapeutic HIV-1 vaccine for vertically-infected infants and children.
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin or Antigen presenting cell</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>B-LCL</td>
<td>B lymphoblastoid cell line</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>E:T</td>
<td>Effector to target ratio</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular cytokine staining assay</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcus enterotoxin B</td>
</tr>
<tr>
<td>Vac</td>
<td>Vaccinia virus</td>
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CHAPTER I
INTRODUCTION

A. Retroviruses.

Retroviruses (family: Retroviridae) are a family of small, enveloped RNA viruses that infect a wide range of organisms throughout nature. The general structural hallmarks of the retrovirus virion are: (1) 2 copies of the viral RNA genome coordinated by nucleocapsid protein surrounded by (2) polymerized capsid and matrix structural proteins that form a spherical or cone-shaped core, (3) a host cell derived lipid membrane that surrounds the nucleoprotein core, and (4) glycoprotein “spike” structures that determine viral tropism and mediate viral entry (1). The genomic structure of all retroviruses contains a common motif: gag – pol – env. Gag encodes several structural proteins such as capsid and matrix. The pol gene encodes the major enzymes utilized by HIV-1 to complete its replicative cycle. Finally, env encodes the envelope-embedded glycoproteins. Retroviruses that contain additional genes are known as complex retroviruses. Retroviral genomes range from 7-12 kb in size, and virions are 80-100nm in diameter.

All retroviruses share several biological features that are uncommonly found among viruses. Foremost among these is the virally-encoded RNA-dependent DNA polymerase, also known as reverse transcriptase (RT). RT is responsible for the generation of a “proviral” double-stranded DNA copy of the retroviral RNA
genome. The discovery of RT not only altered the classical interpretation of the genetic code (i.e. DNA → RNA → protein), but has led to significant improvements in molecular biology technology. Second, retroviruses must integrate their newly-copied DNA genome into a host chromosome. Once integrated, the retroviral genome becomes permanently entrenched in the infected cell's genome, and is passed to daughter cells upon cell division. Finally, retroviruses utilize long terminal repeats, or LTRs, to facilitate integration of the proviral DNA, recruit transcriptional activators and polymerase machinery, and polyadenylate mRNA transcripts.

B. Human immunodeficiency virus type 1.

1. General characteristics and epidemiology.

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that infects cells of the human immune system (1). HIV-1 is a member of the genus Lentiviruses, a group of complex retroviruses that infect primates and many other vertebrates. The HIV-1 genome is 9.2 kb in length. In addition to the canonical gag – pol – env structure characteristic of all simple retroviruses, the HIV-1 genome encodes 6 additional gene products: nef, tat, rev, vpr, vpu, and vif (Figure 1.1). The HIV-1 accessory proteins serve many enzymatic functions, and most are pleiotropic. The organization of the HIV-1 genome is illustrated in Figure 1.2. HIV-1 is not the only known retrovirus to cause disease in humans. For example, human T cell leukemia virus type 1 (HTLV-1), another complex retrovirus
Figure 1.1: HIV-1 virion structure (from Robinson, H.L. New Hope for an AIDS vaccine. 2002 Nature Reviews Immunology 2:239-250.)
causes several adult T cell leukemias (2).

HIV-1 is primarily transmitted through sexual contact, exposure to contaminated blood products, and intravenous drug use. Additionally, HIV-1 may be transmitted from mother to child during pregnancy, birth, or through breastmilk.

HIV-1 was identified in the early 1980s as the causative agent of acquired immunodeficiency syndrome (AIDS) (3, 4). The hallmark of AIDS is the progressive loss of CD4+ T cells during chronic HIV-1 infection and susceptibility to opportunistic pathogens and tumors.

Structural and genetic similarity with the non-human primate lymphotropic lentivirus SIV (simian immunodeficiency virus) suggests that HIV-1 was transmitted from chimpanzees to humans in sub-Saharan Africa. Although the timing of this species jump is unclear, with the advent of transcontinental travel HIV-1 has disseminated to all regions of the world. Developing countries have the highest rates of infection. Sub-Saharan Africa alone accounts for over 70% of all HIV-1 infections. UNAIDS estimated in 2002 that 42 million people worldwide are infected with HIV-1, with over 5 million new HIV-1 infections per year. Infants and children are hit especially hard by HIV-1. Although pediatric HIV-1 infections represent less than 10% of the total number of HIV-1 infections worldwide, 20% of HIV/AIDS-related deaths in 2002 were in infants and children.
**Figure 1.2**: Organization of the HIV-1 genome. (from Greene, W.C., and Peterlin, B.M. 2002 *Nature Medicine* 8:673-680).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function and Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LTR</strong></td>
<td>Long terminal repeat. Contains control regions that bind host transcription factors (NF-kB, NFAT, Sp1, TBP). Required for the initiation of transcription. Contains RNA trans-acting response element (TAR) that binds Tat.</td>
</tr>
<tr>
<td><strong>vif</strong></td>
<td>Viral infectivity factor (p23). Overcomes inhibitory effects of unidentified host factor, yielding more stable RT complexes.</td>
</tr>
<tr>
<td><strong>env</strong></td>
<td>gp160 envelope protein. Cleaved in endoplasmic reticulum to gp120 (SU) and gp41 (TM). gp130 mediates CD4 and chemokine receptor binding, while gp41 mediates fusion. Contains RNA response element (RRE) that binds Rev.</td>
</tr>
<tr>
<td><strong>gag</strong></td>
<td>Pr55Gag Polyprotein processed by PR. MA, matrix (p17). Undergoes glycosylation that helps target Gag polyprotein to endoplasmic reticulum, facilitating integration. CA, capsid (p24). Contains reverse transcriptase, integrase, and ribonuclease H. NC, nucleocapsid (p7). P6, p2, and p1 peptides.</td>
</tr>
<tr>
<td><strong>pol</strong></td>
<td>Polymerase. Encodes a variety of viral enzymes, including PR (p15), RT (p66), and IN (p32). All processed by PR.</td>
</tr>
<tr>
<td><strong>rev</strong></td>
<td>Regulator of viral gene expression (p19). Binds RRE, integrates viral RNA splicing and promotes nuclear export of incompletely spliced viral RNAs.</td>
</tr>
<tr>
<td><strong>tat</strong></td>
<td>Transcriptional activator (p14). Binds TAR in presence of host cyclin T1 and CDK9, enhances RNA Pol II elongation on the viral RNA template.</td>
</tr>
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**Figure 1.2**: Diagram showing the organization of the HIV-1 genome.
2. Viral replication cycle.

The HIV-1 replication cycle may be broken up into several stages. These include entry/fusion, reverse transcription, integration, gene expression and virion production. HIV-1 enters permissive host cells when trimeric gp120/gp41 “spike” molecules engage CD4 on the cell surface. This binding event induces a conformation change within gp120 that exposes a binding domain specific for chemokine receptors, primarily CCR5 and/or CXCR4 (5). Upon binding of the chemokine receptor, coiled coil domains within gp41 fold and expose a fusion peptide that inserts into the host cell membrane (6). This process brings the viral and cellular membranes into close proximity and allows membrane fusion.

Upon virus-cell membrane fusion, the HIV-1 virion core enters the host cell cytoplasm. At this stage, the structure is known as the reverse transcription complex (7). The viral enzyme RT completes a double-stranded DNA copy of the RNA genome as the entire reverse transcription complex is shuttled towards the nucleus on actin filaments. Once reverse transcription is complete, the complex becomes known as the pre-integration complex (8). The primary function of the pre-integration complex is to allow nuclear import of the viral DNA (and associated proteins) in non-dividing cells, such as macrophages (9). While the exact mechanisms that allow nuclear import remain unclear, viral and cellular factors appear to mediate passage of the pre-integration complex through nuclear pores (10, 11).
Once the viral DNA and associated proteins have entered the cell nucleus, integration of the viral genome into the host DNA must take place to allow for expression of viral gene products and replication of the viral genome. The viral enzyme integrase catalyses insertion of the linear viral DNA into the host chromosome. Once accomplished, the "proviral" DNA may either remain transcriptionally silent (i.e. latency), or use viral (tat) and host factors (cyclin T1, NF-κB, NFAT) to allow transcription of viral gene products (12). Complete, unspliced transcripts of HIV-1, as well as singly and multiply spliced mRNA transcripts are produced and are exported to the cytoplasm (13). Splicing of HIV-1 transcripts plays an important role in regulating viral gene expression levels within the infected cell. The viral protein rev plays an important role in the export of several species of viral transcripts by binding to a region of env known as the rev response element (RRE) (14).

Following transcription and translation of HIV-1 mRNAs, several proteins must undergo post-translational modifications. For example, the amino terminus of gag is myristoylated to allow binding to the plasma membrane (15). Additionally, the env protein is heavily glycosylated and thereafter cleaved by a cellular protease to yield gp120 and gp41 (16). As each of the virion components assembles at the plasma membrane, further enzymatic activities allow for the formation and budding of infectious HIV-1 virions to complete the viral replicative cycle.
3. **Permissive host cells and viral tropism.**

HIV-1 is known to productively infect CD4+ T cells, monocytes, macrophages, and dendritic cells, although the vast majority of circulating virus appears to derive from the CD4+ T cell compartment. Other cell types, such as B cells and microglia of the CNS appear to support HIV-1 infection (17), but how these cells contribute to overall HIV-1 replication remains unclear.

Viral tropism, or the type of cells that may be infected by HIV-1, is largely dependent on sequence variation within the viral envelope glycoprotein (env) (18). Viral tropism is most commonly defined by identification of which chemokine co-receptor is used for viral entry. Although HIV-1 may employ other receptors to facilitate viral entry, the chemokine co-receptors CCR5 and CXCR4 are most commonly utilized. As the primary reservoir for HIV-1 replication, CD4+ T cells typically express the chemokine receptor CXCR4 and are infected by “T-tropic” viruses that bind CXCR4. Conversely, macrophages express CCR5 and are infected by “M-tropic” viruses that enter using CCR5. In some individuals, especially those with progressive HIV-1 replication, dual-tropic viruses, those that can bind both CXCR4 and CCR5, may be found (19).

4. **Viral quasispecies and subtypes.**

Due to the high error rate of the HIV-1 RT enzyme, sequence diversity is high, even among circulating viral isolates from single individuals. Viral variants found within an infected individual are termed *quasispecies*. Factors such as cell
tropism, replication fitness, antigenicity, and host cell genetics influence the development of viral quasispecies over time within infected individuals.

Although errors introduced by RT appear to occur randomly throughout the viral genome, not all mutations allow for productive viral replication. As such, conserved regions within the viral genome, those important for structural or enzymatic functions, have been identified. Similarity of sequence within these conserved regions may be used to group HIV-1 isolates documented worldwide into clades, or groups of viruses with shared backbone sequences. Different HIV-1 clades predominate in defined geographical regions. For example, clade C is the predominate HIV-1 subtype found in South Africa whereas clade B HIV-1 is most common in the United States. The variation found in HIV-1 at the level of quasispecies (within individuals) as well as clades (among geographic areas) contributes to the difficulty in developing a vaccine that will have widespread effectiveness.

C. Clinical course of natural HIV-1 infection.

1. Adult HIV-1 infection.

The natural course of HIV-1 infection in adults follows several distinct phases that ultimately result, in the majority of infected individuals, in a severe immunodeficiency syndrome and vulnerability to opportunistic infections (Figure 1.3) (1, 20). Following HIV-1 infection, an acute symptomatic phase lasting several weeks occurs as the virus replicates to very high levels. During primary
viremia, a significant drop in CD4$^+$ T cell counts is typically observed. Adaptive components of the immune response, humoral and cellular, are both primed and develop during this phase. Upon the development of HIV-specific immune responses, primary viremia peaks and declines up to 3 orders of magnitude to establish an equilibrium level of viral replication. This "set-point" of HIV-1 replication reflects the kinetics of viral production from productively-infected cells and viral clearance by host immune responses. The decline in viremia appears to occur with the generation of HIV-specific CD8$^+$ T cell responses (21). The level of HIV-1 viremia that is established at the set-point is prognostic of the rate of progression to the symptomatic phase of chronic HIV-1 infection (22).

Following the acute phase of HIV-1 infection, chronic HIV-1 infection is characterized by a long, asymptomatic period, lasting on average 10 years. Many host cell and viral factors influence the length of asymptomatic HIV-1 infection, including HLA class I genotype, chemokine co-receptor expression patterns and polymorphisms, and viral deletions or mutations that modulate tropism and the "fitness" of viral replication within the host. During this period, CD4$^+$ T cell counts gradually erode and leave the infected individual susceptible to opportunistic infections and tumors when CD4$^+$ T cell counts drop below 200 cells/µL of blood.
Figure 1.3: Natural course of HIV-1 infection in adults. (from Coffin, J., Hughes, S., and Varmus, H. Retroviruses. 1997. Cold Spring Harbor Laboratory Press).
2. Pediatric HIV-1 infection.

As stated previously, HIV-1 may be transmitted from mother to child, either during pregnancy (*in utero*), during labor (peripartum), or through breastmilk. The vast majority of pediatric HIV-1 infections are the result of vertical transmission (i.e. from mother to child) of the virus. In the absence of antiretroviral therapy, approximately 25% of infants acquire HIV-1 from the mother. Transmission rates are higher in developing countries.

Interestingly, the course of primary HIV-1 infection in vertically-infected infants is markedly different than that observed in adults (Figure 1.4). In contrast to the resolution of primary viremia and establishment of a viral set-point as observed in adults, pediatric HIV-1 infection is characterized by sustained high-level HIV-1 replication during the first several years of life (23, 24). Several factors may contribute to high levels of viral replication during the first years of life. First, the overall pool of CD4+ T cells, the main substrate for HIV-1 replication, is larger than that found in adults (25). Second, thymic mass relative to overall body mass is high in infants, and thymocytes have been shown to be susceptible to HIV-1 infection (26). Finally, absent or delayed HIV-specific immune responses may not allow for control of primary viremia (27).

The rapid progression of HIV-1 infection in infants and children contributes to the higher mortality observed in this population. Approximately 15% of untreated HIV-1-infected infants die by 4 years of age.
Figure 1.4: HIV-1 replication in untreated vertical infection (from Shearer, W.T. et al. 1997 N Engl J Med 336 (19):1337-42).
3. Antiretroviral therapy.

Significant advances in the development of antiretroviral therapeutics that target several HIV-1 enzymatic processes have dramatically improved the prognosis of HIV-1-infected individuals in developed countries (28). These include reverse transcriptase, protease, and entry inhibitors. Antiretroviral therapies, especially when used in combination, have greatly improved the prognosis and quality of life of HIV-infected individuals, both adult and pediatric (29, 30). Moreover, administration of therapies during labor has been shown to dramatically lower the rate of vertical transmission. Unfortunately the high cost of these drugs, coupled with poor clinical, social, and political infrastructures in developing nations, have blunted their impact on the HIV-1 epidemic. Furthermore, the development of resistance mutations to each class of antiretroviral therapy available complicates the administration of these drugs and necessitates ongoing development of novel HIV-1 inhibitors. The failure of antiretroviral therapy to stem the growing tide of HIV-1 infections throughout the world underlines the need for the development of prophylactic and therapeutic vaccine strategies to stimulate durable, broad, and potent HIV-specific immunity.

D. T cell immunity during pediatric HIV-1 infection.

1. CD8+ T cell responses.

Human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cell responses have been shown to play an important role in controlling HIV-1 viral replication
during both acute and chronic stages of adult infection (21, 31-33). The use of ART has greatly enhanced the clinical status of many HIV-1-infected individuals, and several studies have indicated that early administration of therapy during the acute phase of viral infection can preserve HIV-1-specific immunity in adults (34).

While the clinical benefit of early ART in vertically-infected neonates is clear, the reduction in HIV-1 viral replication during the initiation of a primary response may impair the generation of HIV-1-specific cellular immunity. There appears to be a lack of persistent HIV-1-specific CD8+ T cell responses in infants who receive early ART (27). In a cross-sectional study, Spiegel et al. have also described reduced CD8+ T cell frequencies in infants and children on ART (35). Potent therapy may reduce the availability of viral antigen for processing and presentation to HIV-1-specific CD8+ T cells thus impairing the generation and expansion of HIV-1-specific memory populations. These populations may be important to help control viral replication during periods of viral breakthrough secondary to poor medication adherence, medication intolerance, or drug resistance.

A better understanding of pediatric CD8+ T cell responses to HIV-1 antigens, as well as how these responses are maintained during the course of ART, may contribute to the development of strategies that boost the quantity and quality of HIV-1-specific CD8+ T cells while still allowing for the continued clinical benefit of ART.
2. CD4\(^+\) T cell responses.

Greater insight into HIV-specific CD4\(^+\) T cells may be particularly important since CD4\(^+\) T cells serve as a major substrate for HIV-1 replication, as well as provide antiviral helper or effector functions (36, 37). Many studies have investigated HIV-specific CD4\(^+\) T cell responses during adult infection, but data regarding pediatric CD4\(^+\) T cell responses are limited. Previous efforts, using lymphoproliferative assays, failed to detect, or detected only low-level CD4\(^+\) T cell responses in vertically-infected infants (27, 38). In general, adult studies have failed to detect vigorous HIV-specific CD4\(^+\) T cell proliferation in individuals with active HIV-1 replication or progressive disease (39, 40), but have detected the presence of HIV-specific CD4\(^+\) T cells that secrete IFN-\(\gamma\) (41-45). Although these studies have made significant contributions to our understanding of HIV-1-specific CD4\(^+\) T cells and their functional responses, the quality, timing, and longevity of pediatric CD4\(^+\) T cell responses remain poorly understood.

E. Thesis Aims.

The objective of this thesis dissertation was to characterize HIV-1-specific T cell immunity in vertically-infected infants and children. As previously discussed, little is known about the timing, magnitude, breadth, and specificity of pediatric cellular immune responses against HIV-1, and how these responses may differ from those generated against other viruses commonly acquired during infancy or from T cell immunity generated in HIV-1-infected adults.
In Chapter 3, the specificity and frequency of HIV-1-specific CD8\(^+\) T cell responses in 17 vertically-infected infants who initiated ART between 1—23 months of age are presented. CMV-specific responses were also followed in 3 young infants coinfected with HIV-1 and CMV. Our data show that CD8\(^+\) T cell responses are less commonly detected in young infants (<6 months of age) than in older infants prior to treatment. Interestingly, CMV-specific responses were detected in several young infants despite low frequency or undetectable HIV-1-specific responses. These findings suggest a specific defect in the generation of HIV-1-specific CD8\(^+\) T cell responses in young HIV-1-infected infants.

To further investigate HIV-1-specific CD8\(^+\) T cell immunity during infancy, several additional methods and reagents were used to characterize CD8\(^+\) T cell responses, viral replication, and viral sequence in a second cohort of HIV-1-infected infants. As illustrated in Chapter 4, HIV-specific CD8\(^+\) T cell IFN-\(\gamma\) responses were detected in the majority of infants prior to initiation of ART using an expanded panel of HIV-1 antigens. CD8\(^+\) T cell IFN-\(\gamma\) responses targeting HIV-1 accessory proteins (nef, tat, rev, vif, vpr) accounted for nearly half of all detectable responses. The hierarchy of responses detected was gag, nef > env > pol, tat, rev, vpr > vif. Quantification of HIV-specific CD8\(^+\) T cell responses also illustrated the importance of HIV-1 accessory proteins in the overall HIV-specific response. To further characterize HIV-specific CD8\(^+\) T cell IFN-\(\gamma\) responses during infancy, a pair of infected twins and an infant with strong HIV-1 specific responses were studied in detail. Data from these experiments reveal the strong
influence of HLA class I genotype as well as viral sequence in shaping the HIV-specific CD8\(^+\) T cell response.

Finally, to address the role of HIV-specific CD4\(^+\) T cell responses during pediatric HIV-1 infection, a large cohort of children was analyzed for the presence of HIV-1 and CMV-specific CD4\(^+\) T cell responses. As presented in Chapter 5, HIV-1-specific CD4\(^+\) T cell responses were commonly detected in the cohort, although they were detected more frequently and at higher intensities in those children with ongoing HIV-1 replication. By contrast, CMV-specific CD4\(^+\) T cell responses were detected most frequently and at higher intensities in those children with undetectable HIV-1 replication, suggesting that ongoing HIV-1 replication had a detrimental impact on the generation and/or maintenance of other virus-specific CD4\(^+\) T cell responses. Analysis of proviral 2-LTR circles revealed that only low levels of HIV-1 replication are required to drive HIV-1-specific CD4\(^+\) T cell responses, suggesting a relationship between HIV-1 replication and the presence of CD4\(^+\) T cell immunity. HIV-1 replication was associated with increased frequencies but impaired \textit{in vitro} proliferative capacity of HIV-specific CD4\(^+\) T cells.

These data have important implications for the treatment and management of HIV-1-infected infants and children, as well as for the design of a vaccine that may allow for improved long-term control of HIV-1 infection.
CHAPTER II
MATERIALS AND METHODS

A. Study populations.

Specific information and data concerning the pediatric study populations studied during the course of thesis research are presented at the beginning of each chapter in the results section. In all cases, written informed consent was obtained from the legal guardian of all study participants in accordance with the Institutional Review Board of the University of Massachusetts Medical School. The guidelines of the U.S. Department of Health and Human Services governing experimentation in humans were followed.

B. Diagnosis of HIV-1 infection.

Diagnosis of HIV-1 infection was made if HIV-1 testing on at least two separate blood samples was positive. In children older than 18 months of age, HIV-1 serology (ELISA with confirmatory Western blot) was used to screen for HIV-1 infection. Many of the children in the cohort were diagnosed younger than 18 months of age, in which case the polymerase chain reaction was used to detect HIV-1 provirus in PBMC. Viral isolation was used to confirm infection in all children.
C. Quantification of plasma HIV-1 RNA copy number by reverse transcriptase (RT)-mediated PCR.

Measurements of plasma viral copy number presented in Chapter 3 were made using the NucliSens quantification assay (Organon Teknika, Durham, NC) according to the manufacturer's instruction. The lower limit of detection is 400 viral RNA copies/mL plasma.

Measurements of plasma viral copy number presented in Chapters 4 and 5 were made using Amplicor quantification assay (Amplicor; Roche Diagnostics, Branchburg, NJ) according to the manufacturer's instruction. Plasma samples with values below the detection limit of the standard assay (<400 copies/mL) were subsequently tested using 450μL of plasma and a modified assay with a detection limit of 50 HIV-1 RNA copies/mL.

All assays were performed in a single laboratory that participates in an ongoing quality certification program for HIV-1 RNA quantitation sponsored by the National Institutes of Health.

D. Diagnosis of CMV infection.

CMV infection status was determined by ELISA for presence of anti-CMV IgG in serum samples taken after 18 months of age. Serological analysis was performed by the University of Massachusetts Serology Laboratory. Congenital CMV infection was diagnosed in infant A13 through CMV urine cultures at <1 mo of age.
E. Peripheral blood and cell lines.

PBMC were isolated from whole blood using the Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation method and viably cryopreserved in RPMI1640 containing 10% DMSO. For use in the ELISPOT assay, cells were thawed and washed twice in RPMI1640 media supplemented with 10% fetal calf serum, 25mM HEPES, and 10mg/L gentamicin (R10 media) prior to counting. Autologous B-LCL were generated for each study participant by EBV/cyclosporin A transformation. B-LCL were maintained in R10 media.

F. Recombinant vaccinia vectors.

Recombinant vaccinia virus vectors containing single HIV-1 clade B gene products (gag [vAbT 141], pol [vCF21], env [vAbT 271], nef [vT23], tat [vT279], rev [vT320], vpr [vT100], and vif [vT28]) were supplied by Therion Biologics (Cambridge, MA). Recombinant vaccinia virus vectors containing the CMV gene products pp65 or gB were supplied by William Britt (University of Alabama, Birmingham). Wild-type vaccinia virus (NYCBH strain; Therion Biologics) was used as a negative control.

For each recombinant vaccinia vector used in the ELISPOT assays, 5x10^5 – 1x10^6 B-LCL cells were infected at MOI = 10 and allowed to incubate 16 –20 hours (overnight) at 37°C in 5% CO₂. Prior to addition to the ELISPOT plate, infected B-LCL were washed twice in R10 media and counted.
G. Detection of Ag-specific CD8+ T cells using a modified ELISPOT assay.

A modified IFN-γ ELISPOT assay was developed based on a previously published method (46). Our assay used autologous B-LCL as antigen-presenting cells to assay for HIV-1-specific release of IFN-γ by antigen-specific CD8+ T cells. Prior to the addition of cells, a 96-well flat bottom plate (MAIPN1450, Millipore, Bedford, MA) was coated with 1mg/mL of anti-IFN-γ mAb (D1K, Mabtech, Nacka, Sweden) and allowed to sit overnight at 4°C. After washing the plate with cold phosphate-buffered saline (PBS), each plate was blocked for non-specific antibody binding by addition of 200 µL/well of R10 media for 2 hours at 37°C. Fifty thousand PBMC from each timepoint were added, in duplicate, for each HIV-1 recombinant vector tested. Ten thousand vaccinia recombinant-infected B-LCL were added to appropriate wells to bring the total volume in each well to 100 µL. This constituted an E:T ratio (PBMC:B-LCL) of 5:1. To ensure assay consistency, all study timepoints (per study subject) and vaccinia recombinant targets were assayed on the same ELISPOT plate. Each plate was incubated 16–20 hours overnight at 37°C in 5% CO2. Following overnight incubation, the plate was washed with cold PBS and 0.5 mg/mL of a secondary, biotinylated anti-IFN-γ mAb (7-B6-1, Mabtech) was added for 3 hours at room temperature. The plate was washed again with cold PBS, and 0.5 mg/mL of a strepavidin/alkaline phosphatase conjugate (SA-ALP, Mabtech) was added for 2 hours at room temperature. Spot-forming cells (SFC) were visualized after alkaline phosphatase color development (BioRad, Richmond, CA).
SFC were counted using a stereoscope (American Optical Corp., Buffalo, NY) at 20x magnification. SFC counts were averaged between duplicate wells to obtain an experimental value for each HIV-1 gene product/PBMC timepoint combination studied. ELISPOT responses are referred to in the text as SFC/10⁶ PBMC over vaccinia background. Two criteria were used to define positive responses. First, each well (from a duplicate pair) must have a minimum of 3 SFC (corresponding to 60 SFC/10⁶ PBMC). Second, the experimental SFC frequency must be 3 standard deviations above the average of all duplicate negative control wells (vaccinia NYCBH) performed on the plate. If both conditions were not met, responses were not considered positive. PMA/ionomycin stimulation of PMBC samples from all study timepoints served as positive controls for IFN-γ expression.

H. Overlapping peptide pools.

To facilitate fine mapping of CD8⁺ T cell epitopes, 121 overlapping peptides spanning HIV-1 gag (clade B, HXB2 isolate) were pooled and used in the ELISPOT assay (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD). Each peptide was 15 a.a. in length, and overlapped adjacent peptides by 11 a.a. To prepare the peptide pools, individual peptides were dissolved at 11 mg/mL in DMSO. An 11x11 matrix was constructed, with each peptide represented in exactly two pools. Equal volumes of 11 peptides were placed into each pool, diluting the concentration of each
individual peptide within the pool to 1 mg/mL. Autologous B-LCL were incubated for 1 hour with 20μg/mL (final concentration per peptide) of each pool for use as antigen-presenting cells in an ELISPOT assay.

I. CD8$^+$ T cell depletion.

Cryopreserved baseline samples on patients A10 and B2 were depleted of CD8$^+$ T cells using magnetic bead separation according to manufacturer's specifications (Dynal ASA, Oslo, Norway). FACS analysis (using anti-CD4$^+$ and anti-CD8$^+$ antibodies) was used to confirm that the small lymphocyte population consisted of less than 4% CD8$^+$ T cells after depletion (Becton Dickenson FACScan). CD8$^+$ T cell depleted and non-depleted PBMC aliquots were then used in the ELISPOT assay as previously outlined.

J. Enumeration of lymphocyte subsets in the peripheral blood.

The relative percentages of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ lymphocytes in the peripheral blood were enumerated by direct immunofluorescence with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated mouse monoclonal antibodies (BD Immunocytometry Systems, San Jose, CA). Samples were analyzed by flow cytometry using CELLQUEST software (FACScan, BD Immunocytometry Systems).
K. Detection of Ag-specific CD4⁺ T cells by intracellular cytokine staining for IFN-γ.

Antigen-specific CD4⁺ T cell responses were detected using an intracellular cytokine staining assay. Briefly, anti-CD28 and anti-CD49d monoclonal antibodies (3µg/mL; BD PharMingen, San Diego, CA) were added to 0.5mL fresh heparinized whole blood aliquots from each study subject and incubated with the following protein antigens: HIV-1SF-2p55 and CMV pp65 (5µg/mL; Austral Biologicals, San Ramon, CA). Two additional whole blood aliquots (both containing the anti-costimulatory antibodies) were set up as negative (no antigen) and positive controls (Staphylococcal enterotoxin B, 4µg/mL; Toxin Technology, Sarasota, FL) for each study subject. Samples were incubated at a 5° slant for 6 hours at 37°C and 5% CO₂. Brefeldin A (BD PharMingen) was added for the final 4 hours of incubation. After incubation, EDTA was added to each tube and samples were fixed (FACS Lysing Solution; BD Immunocytometry Systems) and permeabilized (FACS Permabilizing Solution; BD Immunocytometry Systems) according to the manufacturer's instructions. Each sample was stained with the following conjugated Abs: peridinin chlorophyll-anti-CD4, FITC-anti-CD45RO, phycoerythrin-anti-CD69, and allophycocyanin-anti-IFN-γ (BD Immunocytometry Systems and BD PharMingen). Flow cytometry analysis was performed within 24 hours of sample preparation as described above. Samples were gated on CD4⁺/CD45RO⁺ small lymphocytes and analyzed for CD69 and IFN-γ expression. Results are expressed as the percentage of CD4⁺/CD45RO⁺ small
lymphocytes expressing CD69 and IFN-γ. Antigen-specific CD4\(^+\) T cells were not
detected in the CD45RO\(^-\) population.

All experimental samples exhibited strong CD4\(^+\) T cell IFN-γ responses to
SEB. Responses in the absence of antigen (no antigen controls) ranged from 0—
0.05% (mean: 0.02%). Experimental responses were considered positive if they
exceeded 0.06% (two standard deviations above the mean negative control
value).

After stimulation with HIV-1 p55 antigen, CD4\(^+\) T cell IFN-γ responses in HIV-
1-uninfected adults (median: 0.01%; range: 0.01—0.03%) did not differ from
background responses (no antigen control; median: 0.01%; range: 0—0.04%).
Similarly, CMV pp65-specific CD4\(^+\) T cells were detected in two HIV-1-
uninfected, CMV seropositive donors, but were not detected in CMV
seronegative donors.

L. Lymphoproliferative assays.

Lymphoproliferative assays were performed as previously described (27, 39,
44) with the following modifications. PBMC were separated from fresh whole
blood by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density
centrifugation and were incubated with HIV-1\(_{SF-2}\) p55 and CMV pp65 protein
antigens at a concentration of 10\(\mu\)g/mL (Austral Biologicals). PBMC were
incubated in triplicate wells (10\(^5\) cells/well) for 7 days before harvesting. Cells
incubated with media alone or pokeweed mitogen (PWM; 10\(\mu\)g/mL; Sigma-
Aldrich, St. Louis, MO) served respectively as negative and positive controls. Plates were analyzed (Wallac 1450 MicroBeta Trilux; PerkinElmer Wallac, Gaithersburg, MD) and stimulation indices (SI) were defined as the ratio of the mean counts per minute of the stimulated well to the mean counts per minute of the negative control wells. Assays were considered valid only if the counts per minute measured in each of the negative control wells were less than 1,000. Stimulation indices greater than 3 were considered positive responses.

*M. Detection of HIV-1 2-LTR circles in PBMC.*

PBMC pellets were resuspended in buffer P1 and extrachromosomal DNA was purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) using the modifications recommended by the manufacturer for the isolation of low copy number plasmids. Final elution of the purified DNA was in 120μL elution buffer preheated to 70°C. 2-LTR junction sequences were amplified from 30μL of extrachromosomal DNA in a 50μL reaction containing 1X HotStarTaq buffer, 200μM deoxynucleotide triphosphates, 500nM each primer, 200nM fluorogenic probe and 2U of HotStarTaq (Qiagen). The reverse and forward primers were 5'-TAGACCAGATCTGAGCCTGGGA-3' and 5'-GTAGTTCTGCCAATCAGGGAAG-3', respectively, which annealed to nucleotides 13 to 34 (HIV-1 LTR R region) and nucleotides 8770 to 8749 (HIV-1 LTR U3 region) of HIV-1LAI (Genbank accession number K02013). The fluorogenic probe (5'-AGCCTCAATAAGCTTGCCATGTC-3') was complementary to nucleotides
67-93 of HIV-1\textsubscript{LAI} and was modified with 6-FAM (6-carboxyfluorescein) reporter dye on the 5' end and 6-TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3' end. After an initial denaturation/enzyme activation step (95°C, 10 min), PCR amplification proceeded for 45 cycles (95°C, 15s; 62°C, 1 min). To control for the effect of sequence polymorphisms at primer binding sites and to allow for the quantitation of total viral genomes, amplifications were performed using primers that were reversed in orientation to those listed above and a different probe (5'-6-FAM-AGTGGCGAGCCCTCAGTGCTGC-6-TAMRA-3') which annealed to nucleotides 9103-9081 of HIV-1\textsubscript{LAI}. Copy numbers were determined from a standard curve generated by the quantitation software of the ABI Prism 7700 sequence detection system. The assay limit of detection was 1 copy.

\textit{N. Generation and use of HLA class I (A*0201) tetramers.}

Tetramers of HLA A*0201 bound to the gag\textsubscript{p17} peptide SL9 (a.a. 77-85, SLYNTVATL) were generated as previously described (27). Briefly, HLA A*0201 heavy chain, \(\beta_2\)-microglobulin, and SL9 peptide were refolded (monomer) and biotinylated. Tetramers were formed by the addition of a streptavidin-allophycocyanin conjugate (BD PharMingen).

Fresh whole blood samples or viably cryopreserved PBMC were stained with 1\(\mu\)L of tetramer as well as 10\(\mu\)L each of anti-CD3-PerCP (BD Immunocytometry Systems) and anti-CD8-FITC (Sigma-Aldrich) to allow discrimination of epitope-
specific CD8$^+$ T cells. Samples were fixed (FACS Lysing Solution; BD Immunocytometry Systems), washed, and analyzed within 24 hours.

O. Sequence analysis of CD8$^+$ T cell epitopes in HIV-1 gag.

HIV-1 RNA was isolated from patient plasma samples using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). cDNA was generated using random hexamers or a reverse primer specific for the 5' end of pol (RTRD: 5'-CTGTCCACCATGCTTCCC-3') to allow for complete amplification of the gag gene (GeneAmp RNA PCR Kit; Applied Biosystems, Branchburg, NJ). Following cDNA synthesis, PCR reactions were used to amplify the gag SL9 (a.a. 77-85; forward primer: 5'-CAGTATTAAGCGGGGGAGAATT-3', reverse: 5'-CCACTGTGTTTAGCATGG-3') and VV9 (a.a. 362-370; forward primer: 5'-ATGAGAGAACCAAGGGGAAGTGA-3', reverse: 5'-TGTTGGCTCTGTCTGCTCT-3') epitopes (HotStarTaq DNA Polymerase; Qiagen). A second, nested PCR reaction was performed to further amplify these regions (SL9, forward primer: 5'-GTATGGGCAAGCGGGAGTGAATT-3', reverse: 5'-CTGTGTGGTTCCTTGTGTCAGC-3'; VV9, forward primer: 5'-ATAATCCACCTATCCAGGGAGAAAT-3', reverse: 5'-CTTTGCCAACATTGAAACACTT-3'). Following verification of specific amplification by gel electrophoresis, the gag amplicons were ligated into pCR2.1 plasmids and transformed into INVαF' cells (TA Cloning Kit; Invitrogen, Carlsbad, CA). Clones were selected (β-gal) after overnight incubation on ampicillin-infused
LB plates. Plasmid DNA was amplified overnight in kanamycin-infused slant cultures and isolated using the QIAprep Spin Miniprep Kit (Qiagen). Following restriction digest analysis, clones were amplified (Platinum Pfx DNA Polymerase; Invitrogen) and purified (QIAquick PCR Purification Kit; Qiagen). A Big Dye 3.0 sequencing PCR reaction was performed using multiple primers spanning the gag gene, or single primers specific to epitope/regions of interest (Applied Biosystems). After ethanol precipitation, all samples were sequenced by the University of Massachusetts Center for AIDS Research Sequencing Facility.

P. Statistical analysis.

All data sets were tested for statistical differences using the Wilcoxon Rank Sum (univariate) or Kruskal-Wallis Rank Sum (multivariate) nonparametric tests for non-Gaussian distributed data. All tests were two-tailed and p-values equal to or less than 0.05 were considered significant. All analyses were performed using Axum 7.0 software (MathSoft, Cambridge, MA).
CHAPTER III

Infrequent Detection of HIV-1-Specific, but not Cytomegalovirus-Specific, CD8+ T Cell Responses in Young HIV-1-Infected Infants.

A. Study population characteristics.

Seventeen infants with vertical HIV-1 infection were studied prior to and following combination antiretroviral therapy. All but one infant were treated with zidovudine, lamivudine, and ritonavir and were participants in the Pediatric AIDS Clinical Trials Group (PACTG) Protocol 345, a clinical trial designed to evaluate the pharmacokinetics of ritonavir in young infants and the virologic and immunologic consequences of early therapy. Infant A13 received stavudine, lamivudine, nevirapine, and nelfinavir through PACTG Protocol 356. Inclusion in the present study was based solely on PBMC sample availability. Clinical and immune status, viral loads, and response to therapy in our study population did not differ from the trial group as a whole.

Characteristics of the study population are presented in Table 3.1. Age at study entry ranged from 1 month to 23 months. Subjects were divided into two groups based on age at therapy initiation: <6 months of age (group A, n=13; mean age: 2.9 months) and >6 months of age (group B, n=4; mean age: 17.6 months). PBMC samples from study weeks 0 (baseline), 8 or 12, 24 or 28, and 48 were employed in the analysis. Baseline CD4+ T cell counts were <25% in
Table 3.1

<table>
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<th>Age at baseline (mo)</th>
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<th>%CD4+ T cells at baseline</th>
<th>Plasma Viral Load at Baseline</th>
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Table 3.1: Study population characteristics. Seventeen infants with vertically-acquired HIV-1 infection (groups A and B). Six HIV-uninfected, age-matched infants (group C) and 4 HIV-infected infants and children (group D) served as control subjects.
only 2 infants. Six uninfected infants born to HIV-1-infected women, in the same age range as the 17 study infants, were used as control subjects (group C; age range: 2-24 months, mean age: 7.6 months). Samples from four children (group D; age range: 14-65 months, mean age: 44 months) with established HIV-1 infection were used to validate the B-LCL ELISPOT assay.

Seven infants were classified as virologic responders (R) as plasma viral load fell to undetectable levels (<400 copies/mL) by 16 weeks on therapy and remained undetectable at all subsequent timepoints. An additional 6 infants controlled viral replication by week 16 but failed to keep viral replication below 400 copies/mL for the entire course of the study and were coded as incomplete responders (IR). The remaining 4 non-responder (NR) infants did not control viral replication.

B. Autologous B-LCL can be used as antigen-presenting cells in an ELISPOT assay.

Previous studies using limiting dilution assays or non-specific in vitro stimulation have shown that cytotoxic T cell responses are uncommonly detected in the PBMC of young vertically-infected infants (47, 48). The IFN-γ ELISPOT assay has been widely used to detect functional antigen-specific CD8⁺ T cell responses after minimal in vitro stimulation in several viral systems. To increase the chances of detecting HIV-1-specific responses in the present study using the ELISPOT assay, a panel of 4 recombinant vaccinia vectors carrying HIV-1 gag,
pol, env, and nef were used. This strategy avoids limiting the breadth of detected responses to previously defined class I HLA-restricted HIV-1 epitopes.

After evaluation of this assay system using adult PBMC (data not shown), PBMC from 4 HIV-1-infected children (group D) were tested (Fig. 3.1a). IFN-γ ELISPOT responses were detected in all 4 children against HIV-1 gag (range: 240-1480 SFC/10^6 PBMC, mean: 750 SFC/10^6 PBMC). Env-specific responses (range: 230-770 SFC/10^6 PBMC, mean: 580 SFC/10^6 PBMC) were also detected at similar levels in 3/3 children studied. While the response to vaccinia alone was 180 SFC/10^6 PBMC in one child (D2), all other background responses were low (60 SFC/10^6 PBMC or less; representing <3 SFC per well at 5x10^4 PBMC per well).

C. HIV-1-specificity of CD8+ T cell responses.

To confirm that IFN-γ ELISPOT responses detected against these HIV-1 recombinants were HIV-1-specific, 6 uninfected infants (group C) ranging in age from 2-24 months were also studied. Responses to HIV-1 gene products were not detected in any of the uninfected infants (Fig. 3.1b).

D. HIV-1-specific CD8+ T cell responses prior to antiretroviral therapy.

Prior to the initiation of antiretroviral therapy, IFN-γ responses to HIV-1 gene products were detected in only two infants (15%) < 6 months of age (Fig. 3.2). Infant A5 generated a low-level response to nef (80 SFC/10^6 PBMC). Infant
Figure 3.1

A.

B.
**Figure 3.1:** Detection of HIV-1-specific IFN-γ responses using a modified ELISPOT assay. (a) IFN-γ responses were detected in four HIV-1-infected children with established infection against HIV-1 gag and env. (b) Uninfected, age-matched infants did not generate IFN-γ ELISPOT responses against a panel of HIV-1 recombinant vaccinia vectors. All responses are listed as SFCs per million PBMCs. Single time points were tested for HIV-1-specific responses in each subject. Blank spaces indicate experiments not performed.
A10 generated responses to both the env and nef gene products (720 and 700 SFC/10^6 PBMC, respectively). Conversely, IFN-γ responses to at least a single HIV-1 gene product were detected at baseline in all 4 infants (100%) >6 months of age (group B). Three of four older infants generated responses against single HIV-1 gene products (pol=2, nef=1), while responses to gag (920 SFC/10^6), pol (100 SFC/10^6), and env (100 SFC/10^6) were detected in one infant (B2). Interestingly, infant B2 presented with the lowest baseline plasma viral load of the 17 study participants (1700 copies/mL plasma). The difference in the detection of HIV-1-specific IFN-γ responses between the two age groups was highly significant (P<0.005) by Fisher's Exact test. Overall baseline IFN-γ ELISPOT responses were directed against only one HIV-1 gene product in 4/6 infants (67%; infants A5, B1, B3, and B4) who generated detectable responses prior to therapy initiation. The hierarchy of responses among all infants was nef/pol (n=3) > env (n=2) > gag (n=1). Background responses to vaccinia were low in all infants and ranged from 0-60 SFC/10^6 PBMC (mean: 9 SFC/10^6 PBMC).

E. HIV-1-specific CD8^+ T cell responses following the initiation of antiretroviral therapy.

To investigate the ability of vertically-infected infants to generate and maintain responses during combination antiretroviral therapy, the 17 infants were followed longitudinally through 48 weeks. In both young infants (A5 and A10) with
Figure 3.2

A. Vaccinia

B. Gag

C. Pol

D. Env

E. Nef
Figure 3.2: HIV-1-specific IFN-γ ELISPOT responses in young infants prior to and during ART. IFN-γ ELISPOT responses are shown for all study infants against wild-type vaccinia virus (NYCBH) (a), HIV-1 gag (b), HIV-1 pol (c), HIV-1 env (d), and HIV-1 nef (e). Virologic responder status is indicated as outlined in Table I. Asterisks indicate significant responses as defined in Chapter 2. Blank spaces indicate experiments not performed.
became undetectable following control of viral replication. It should be noted that both infants were classified as virologic responders and had undetectable plasma viral loads by 8 weeks following therapy initiation.

Intermittent low-level IFN-γ ELISPOT responses were detected in 6 group A infants over the course of antiretroviral therapy. Infants A2, A6, A7, A8, A9, and A13 generated responses against gag, pol, and nef with a mean of 130 SFC/10^6 PMBC (range 60 - 240 SFC/10^6 PBMC). Only one of these infants was classified as a virologic responder (A13) and 4/6 had detectable plasma HIV-1 RNA at the time of the positive IFN-γ ELISPOT response.

IFN-γ ELISPOT responses were again detected at subsequent timepoints in all 4 of the older infants (group B) following therapy. In 3/4 infants (B1, B2, and B3) these responses were directed at the same HIV-1 gene products as detected at baseline. Two of these three infants (B1 and B3) broadened the IFN-γ ELISPOT response to include a second HIV-1 gene product (in both cases, env). Gag-specific ELISPOT responses were detected through week 8 in infant B2: however, pol and env responses were not maintained. Pol responses were not detected after therapy in infant B4; however an intermittent response to gag was detected at week 24 of therapy. In 3 of 4 infants, ELISPOT responses were detected after the initiation of ART, even following the control of viral replication (B1).

\[ \text{F. CD8}^+ \text{ T cells mediate the IFN-γ response.} \]
As described above, vigorous IFN-γ responses were detected in the baseline PBMC samples of patients A10 (env and nef) and B2 (gag). CD8+ T cell depletions were performed to determine which T cell population mediated these responses. Baseline PBMC samples from both infants were divided into two fractions, one of which was depleted of CD8+ T cells by magnetic separation. FACS analysis confirmed that CD8+ T cells represented <4% of the total small lymphocyte pool after depletion. The ELISPot assay was used to enumerate IFN-γ responses in both the CD8+ and CD8- PBMC fractions for both patients. Positive responses, against env and nef by infant A10, and against gag by infant B2, were again detected only in the CD8+ fractions (data not shown). IFN-γ responses were not detected in the CD8- fraction of either infant.

G. Detection of CMV-specific CD8+ T cell responses despite low or absent HIV-1-specific responses.

Three young infants (A9, A12, A13) coinfected with HIV-1 and CMV were evaluated to determine whether CMV-specific CD8+ T cell responses were generated prior to and during the course of ART (Fig. 3.3). Recombinant vaccinia viruses encoding the CMV late gene products pp65 and gB were used with autologous B-LCL in an ELISPot assay. CMV pp65-specific responses were detected in two infants, A9 and A13, prior to and during antiretroviral therapy (range: 100-240 SFC/10^6 PBMC). Baseline CMV responses in infant A12 were not tested; however at weeks 8 and 48 on therapy, pp65-specific (200 and
Figure 3.3
Figure 3.3: Detection of CMV-specific CD8$^+$ T cell responses in three young HIV-1 and CMV coinfected infants. IFN-γ ELISPOT responses detected in infants A9, A12, and A13 against wild-type vaccinia virus (NYCBH) (a), CMV pp65 (b), and CMV gB (c). Asterisks indicate significant responses. Blank spaces indicate experiments not performed.
340 SFC/10^6 PBMC, respectively) and gB-specific (80 and 60 SFC/10^6 PBMC, respectively) responses were detected. Responses to either CMV gene product were not detected in the PBMC of 2 CMV seronegative infants (data not shown).

H. Discussion.

A modified IFN-γ ELISPOT, using autologous B-LCL as antigen-presenting cells, has facilitated the detection and enumeration of low frequency CD8^+ T cell-restricted responses to several HIV-1 gene products. This approach is well suited for studying CD8^+ T cell responses in infants and children since it required only small numbers of PBMC to evaluate CD8^+ T cell-restricted responses to 8 HIV-1 gene products. By maintaining a consistent E:T ratio between PBMC and B-LCL (as well as the infecting MOI), antigen presentation is better standardized when compared to direct infection of PBMC cultures. Finally, low background responses by infants to vaccinia and the EBV-transformed B-LCL made this assay particularly well suited to the study of pediatric samples.

In this chapter, HIV-1-specific CD8^+ T cell responses were studied in 17 HIV-1 vertically-infected infants prior to and during the course of ART. The data demonstrate that HIV-1 CD8^+ T cell responses were less commonly detected in younger infants (<6 months of age) than in older infants (>6 months) prior to ART. Following the initiation of ART, HIV-1-specific CD8^+ T cell responses were uncommonly detected in infants with persistent control of viral replication. Finally, CMV-specific CD8^+ T cell responses were detected in 3 HIV-1/CMV coinfected
infants that lacked HIV-specific CD8+ T cell responses. These results indicate that young vertically-infected infants can generate and maintain CD8+ T cell IFN-γ responses, and that host and/or viral factors may specifically impede the development of early HIV-specific responses in some infants.
CHAPTER IV

Importance of HIV-1 Accessory Gene Products and the Role of Epitope Escape Mutations in Early Pediatric HIV-1 Infection.

A. **Study population characteristics.**

Thirteen infants with vertical HIV-1 infection were studied prior to initiation of combination antiretroviral therapy. All but three infants (P-1042, P-1043, and P-1115) were treated with zidovudine, lamivudine, and nevirapine and were participants in the Pediatric AIDS Clinical Trials Group (PACTG) Protocol 356, a clinical trial designed to evaluate the pharmacokinetics of ritonavir in young infants and the virologic and immunologic consequences of early therapy. Inclusion in the present study was based solely on PBMC sample availability. Clinical and immune status, viral loads, and response to therapy in our study population did not differ from the trial group as a whole.

Characteristics of the study population are presented in Table 4.1. The median age at study entry was 2.7 mo (range: 1.2—4.8 mo). The median CD4⁺ T cell count at study was 2641 cells/μL (range: 1,370—4,606 cells/μL). All infants presented with high levels of HIV-1 replication (median: 170,672 copies/mL; range: 16,721—2,361,810 copies/mL). PBMC samples drawn prior to the initiation of therapy were employed in the cross-sectional analysis. PBMC and plasma samples donated during the course of therapy were also used.
Table 4.1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (mo)</th>
<th>CD4⁺ T cell count (cells/µL)</th>
<th>Plasma HIV-1 RNA (copies/mL)</th>
<th>HIV-specific CD8⁺ T cell responses</th>
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<tr>
<td>P-1210</td>
<td>1.8</td>
<td>2,472</td>
<td>135,417</td>
<td>None detected</td>
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<td>P-1316</td>
<td>3.4</td>
<td>2,641</td>
<td>97,798</td>
<td>None detected</td>
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<tr>
<td>P-1327</td>
<td>3.3</td>
<td>2,407</td>
<td>1,128,889</td>
<td>gag, vif</td>
</tr>
<tr>
<td>P-1262</td>
<td>3.4</td>
<td>2,972</td>
<td>2,361,810</td>
<td>gag, pol, env, nef, vpr</td>
</tr>
<tr>
<td>P-1217</td>
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<td>2,328</td>
<td>569,813</td>
<td>None detected</td>
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<td>P-1042</td>
<td>2.7</td>
<td>2,640</td>
<td>370,279</td>
<td>gag, nef, tat</td>
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<td>P-1043</td>
<td>2.7</td>
<td>2,681</td>
<td>399,943</td>
<td>rev</td>
</tr>
<tr>
<td>P-1115</td>
<td>3.2</td>
<td>1,370</td>
<td>141,979</td>
<td>gag, pol, env, nef, vpr</td>
</tr>
<tr>
<td>P-1223</td>
<td>1.2</td>
<td>4,606</td>
<td>170,672</td>
<td>env</td>
</tr>
<tr>
<td>P-1244</td>
<td>2.4</td>
<td>4,085</td>
<td>51,582</td>
<td>gag, nef, tat, rev</td>
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<td>P-1247</td>
<td>1.3</td>
<td>3,467</td>
<td>16,721</td>
<td>nef</td>
</tr>
<tr>
<td>P-1252</td>
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<td>2,187</td>
<td>217,906</td>
<td>None detected</td>
</tr>
<tr>
<td>P-1235</td>
<td>2.1</td>
<td>4,836</td>
<td>83,169</td>
<td>None detected</td>
</tr>
<tr>
<td>Median</td>
<td>2.7</td>
<td>2,641</td>
<td>170,672</td>
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</tbody>
</table>

Table 4.1: Population characteristics at study entry. Age, CD4⁺ T cell count, HIV-1 load, and CD8⁺ T cell responses are listed for 13 vertically-infected infants.
for longitudinal analysis of CD8\(^+\) T cell responses, viral replication, and viral sequence analysis in infants P-1042, P-1043, P-1115.

**B. HIV-specific CD8\(^+\) T cell responses detected in the majority of vertically-infected infants.**

Previous studies by our group and others have found that HIV-specific CD8\(^+\) T cell responses are rarely detected in untreated, vertically-infected infants <6 mo of age (27, 35, 49). It is unclear if the lack of detectable HIV-specific CD8\(^+\) T cell responses is an accurate reflection of the *in vivo* immune state during infancy, or if the methodologies employed fail to detect weak or obscure HIV-specific CD8\(^+\) T cell responses.

In the present study, a large panel of recombinant HIV-1 antigens was used to increase our ability to detect HIV-specific CD8\(^+\) T cell IFN-\(\gamma\) responses in young infants prior to the initiation of ART. The new panel added 4 HIV-1 gene products, the accessory proteins tat, rev, vif, and vpr, to our existing panel of vaccinia recombinants encoding gag, pol, env, and nef. Aside from the tat and rev recombinants (based on viral sequence isolated from patient P-1042), all recombinants were based on either consensus B or common clade B lab strains (Table 4.2). These vectors were used to infect autologous B-LCL for use as target cells in an ELISPOT assay. This approach is not limited by disparities between patients with respect to HLA class I or antigen processing and presentation capability. HIV-specific CD8\(^+\) T cell responses were detected in 8 of
13 (62%) infants prior to initiation of ART (Figure 4.1). Among responders, the HIV-specific CD8+ T cell response was broad (median: 2.5 gene products recognized), and each HIV-1 gene product in the panel was recognized by at least one infant. The median frequency of IFN-γ-secreting CD8+ T cells among responders was 270 SFC/10^6 PBMC (range: 80—5160 SFC/10^6 PBMC), consistent with previously reported data from our group and others. No relationships between the presence of HIV-specific CD8+ T cell responses and age, CD4+ T cell count or percentage, or plasma HIV-1 load were observed.

C. Hierarchy of HIV-specific CD8+ T cell responses and recognition of HIV-1 accessory gene products.

The relative contribution of each HIV-1 gene product in the overall HIV-specific CD8+ T cell response is illustrated in Figure 4.2. The hierarchy among the 22 HIV-specific CD8+ T cell responses detected in the 8 responder infants was gag, nef (5) > env (3) > pol, tat, rev, vpr (2) > vif (1). Median HIV-specific CD8+ T cell responses ranged from 140—700 SFC/10^6 PMBC against single HIV-1 gene products. Consistent with previous studies in adults and children, gag-specific CD8+ T cell responses occur most frequently and at the highest intensity.

Despite the relatively small size of the HIV-1 accessory proteins compared to the more frequently studied structural proteins, responses against HIV-1 nef, tat, rev, vif, and vpr accounted for over half of the total HIV-specific CD8+ T cell
Table 4.2

<table>
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<tr>
<th>Vector</th>
<th>Gene Product</th>
<th>Background/Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>vAbT 141</td>
<td>gag (p55)</td>
<td>Clade B / BH-10</td>
</tr>
<tr>
<td>vCF21</td>
<td>pol</td>
<td>Clade B / HXB2</td>
</tr>
<tr>
<td>vAbT 271</td>
<td>env</td>
<td>Clade B / BH-10</td>
</tr>
<tr>
<td>vT23</td>
<td>nef</td>
<td>Clade B / NL4.3</td>
</tr>
<tr>
<td>vT279</td>
<td>tat</td>
<td>P-1042 isolate</td>
</tr>
<tr>
<td>vT320</td>
<td>rev</td>
<td>P-1042 isolate</td>
</tr>
<tr>
<td>vT100</td>
<td>vpr</td>
<td>Clade B / IIiB</td>
</tr>
<tr>
<td>vT28</td>
<td>vif</td>
<td>Clade B / IIiB</td>
</tr>
</tbody>
</table>

Table 4.2: Recombinant vaccinia viruses encoding single HIV-1 gene products.

All vectors courtesy of Therion Biologicals Inc., Cambridge, MA.
Antigen-specific CD8+ T cell responses

Patient ID

Vaccinia
Gag
Pol
Env
Nef
Tat
P85
Vpr
Vif
pp65

SFC/10^6 PBMC

Figure 4.1
Figure 4.1: HIV-1-specific CD8+ T cell responses were measured using a modified ELISPOT assay in 13 infants prior to initiation of ART. Responses are recorded as spot-forming cells (SFC) per million PBMC. Asterisks indicate responses that met two criteria for positivity: 1.) duplicate wells must have at least 60 SFC/10^6 PBMC, and 2.) The mean SFC/10^6 PBMC frequency between duplicate wells must be greater than 2 times the standard deviation of the mean background level (vaccinia virus control). Responses against the CMV tegument protein pp65 are shown in several infants.
response (Figure 4.3). Twelve of 22 responses (55%) were directed against the accessory gene products. In addition, the intensity of responses directed against accessory gene products was also considerable - 38% (5380 of 14340 SFC detected) of the total number of HIV-specific CD8+ T cells detected. Two factors may have contributed to an underestimation of the accessory gene component of the HIV-specific response in our study. First, the potent gag-specific CD8+ T cell response detected in P-1115 accounted for over a third of the total HIV-specific SFC count (5160 of 14340 total SFC). Indeed, if this response is removed from consideration, the contribution of responses against nef, tat, rev, vif, and vpr rises to 59% of the total HIV-specific CD8+ T cell response (5380 of 9180 SFC). Second, due to the small amounts of PBMC obtained from these infants, vif and vpr-specific responses were not measured in 3 responder infants (P-1042, P-1043, and P-1244).

D. Divergent HIV-1 epitope-specific CD8+ T cell responses in infants with similar HLA haplotypes.

Two infants in the cohort, P-1042 and P-1043, are fraternal twins who appear to have acquired HIV-1 during delivery. Despite sharing half of their HLA class I alleles, as shown in Table 4.3, twins P-1042 and P-1043 generated quite dissimilar HIV-1-specific CD8+ T cell IFN-γ responses. Responses against tat, gag, and nef were detected in infant P-1042 whereas a single rev-specific response was detected in P-1043.
Figure 4.2

A.

![Bar graph showing the number of infants with detectable CD8 T-cell responses for different HIV-1 gene products. The gene products include Gag, Pol, Env, Nef, Tat, Rev, Vif, and Vpr.]

B.

![Bar graph showing the median HIV-specific CD8 T-cell frequency in 9 infants for different HIV-1 gene products. The gene products include Gag, Pol, Env, Nef, Tat, Rev, Vif, and Vpr.]
**Figure 4.2:** Breadth and intensity of HIV-1-specific CD8+ T cell IFN-γ responses in 8 vertically-infected infants. Twenty-two HIV-specific CD8+ T cell responses were detected against a panel of 8 HIV-1 gene products. (a) The number of CD8+ T cell responses directed against each HIV-1 gene product by the 8 responder infants. (b) The median number of HIV-specific CD8+ T cell responses, as measured by ELISPOT analysis, directed against each HIV-1 gene product by the 8 responder infants.
To further investigate these responses, epitope mapping experiments using the ELISPot assay were performed in patient P-1042. Although responses directed against vectors encoding lab strain HIV-1 antigens were detected in P-1042 (i.e. gag BH-10 and nef NL4.3), the most prominent response was directed against the vaccinia recombinant vector encoding an autologous tat sequence isolated shortly after birth. Pools of overlapping peptides spanning the tat protein and fine mapping using truncated peptides revealed that this early tat-specific response is focused on a single epitope (tat a.a. 35-44; QACFTTKAL; data not shown), restricted by the HLA class I Cw*0303 allele. Interestingly, the sequence of this optimal epitope differs from the more than 35 published clade B isolates (50). Rather, the majority of isolates contain 3 amino acid differences, including an A→V change at the important 2nd a.a. "anchor" residue. It is unclear whether these polymorphisms would stimulate the QL9-specific CD8+ T cells in P-1042, or if HLA Cw*0303 can bind and present these peptides. This result raises the possibility that, had not autologous viral sequences been used as the stimulating antigen, these tat-specific CD8+ T cells would have remained undetected.

E. Longitudinal analyses reveal differential recognition of CD8+ T cell epitopes during early HIV-1 infection in P-1115.

To examine the development and longevity of HIV-specific CD8+ T cells during infancy, HIV-specific CD8+ T cell responses were studied in greater detail
Figure 4.3

A.

B.
Figure 4.3: Contribution of each HIV-1 gene product to the CD8+ T cell response in 8 responder infants. The number of SFC (per $10^6$ PBMC) detected against each HIV-1 gene product were totaled and used to compare the intensity of the CD8+ T cell responses against each HIV-1 gene product. (a) 22 responses against 8 HIV-1 gene products. (b). Separate analysis with intense gag-specific response detected in P-1115 removed from consideration (see text).
in infant P-1115. Longitudinal analysis in this infant was attractive due to the early and potent HIV-specific CD8\(^+\) T cell responses detected prior to the initiation of ART. ELISPOT analysis revealed that HIV-specific CD8\(^+\) T cell responses directed at gag, pol, env, were maintained during the first year of life, despite significant decreases in plasma viral loads concurrent with ART initiation (Figure 4.4). Since this infant expresses the common HLA class I A*02 allele, longitudinal PBMC samples were stained with HLA class I tetramers specific for the immunodominant SL9 epitope found in gag\(_{p17}\) (Figure 4.5). Interestingly, SL9-specific CD8\(^+\) T cells were not detected until the second year of life in the PBMC of infant P-1115, suggesting that an alternative gag epitope (or epitopes) mediated the early gag-specific CD8\(^+\) T cell response.

To identify the epitope(s) targeted during the early gag-specific CD8\(^+\) T cell response in P-1115, pools of overlapping peptides spanning the gag protein were used in an ELISPOT analysis (Figure 4.5). PBMC drawn 6 weeks following therapy initiation revealed a highly focused response directed at peptide #91, a peptide spanning the p24/p2 cleavage site within gag. Fine mapping using truncated peptides revealed that the response was directed at the previously identified VV9 epitope (VLAEMSQV), restricted by HLA A*02 (data not shown). Confirming the HLA tetramer analysis, a SL9-specific CD8\(^+\) T cell response was detected in PBMC drawn 77 weeks following therapy initiation. These data indicate that a significant shift in epitope dominance and usage occurred during the end of the first year of life in infant P-1115. Early CD8\(^+\) T cell responses
### Table 4.3

<table>
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<th>Patient</th>
<th>HLA A</th>
<th>HLA B</th>
<th>HLA C</th>
</tr>
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<tbody>
<tr>
<td>P-1042</td>
<td>A*1101; 2602</td>
<td>B*3901; 5101</td>
<td>Cw*0303; 1203</td>
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<tr>
<td>P-1043</td>
<td>A* 2602; 3002</td>
<td>B*1801; 3901</td>
<td>Cw*0501; 1203</td>
</tr>
</tbody>
</table>

**Table 4.3**: Class I HLA genotype for twins P-1042 and P-1043. Shared alleles are in bold.
Figure 4.4

A.

B.
Figure 4.4: Longitudinal HIV-specific CD8\(^+\) T cell responses in infant P-1115. (a) The ELISPOT assay reveals strong gag-specific CD8\(^+\) T cell IFN-\(\gamma\) responses during the first year on therapy. (b) A classically-defined A*0201-restricted CD8\(^+\) T cell response against the gag SL9 epitope is absent during the first year of life, but quickly develops into the dominant gag-specific CD8\(^+\) T cell response.
targeted only the gag VV9 epitope during the first year of life. Thereafter, the SL9 epitope was the predominant target of the gag-specific CD8⁺ T cell response.

F. Role of viral escape mutations during early pediatric HIV-1 infection.

One potential explanation for the absence of SL9-specific CD8⁺ T cells during the first year of life in infant P-1115 is the presence of escape mutations within CD8⁺ T cell epitopes. It is possible that escape mutations could have been transmitted to the infant from its mother, who shares at least half of her HLA class I allotype with P-1115. To test this hypothesis, viral sequences spanning the SL9 epitope within the gag gene were obtained at 4 timepoints that span the first two years of ART administration in patient P-1115. As presented in Table 4.4, viral sequence analysis of the SL9 epitope failed to reveal any deviation from the "wild-type" SLYNTVATL epitope. Given the lack of non-synonymous mutations within the SL9 epitope, it appears unlikely that the absence of SL9-specific CD8⁺ T cell responses during the first year of life can be attributed to an epitope escape mechanism. Interestingly, wild-type sequences persisted even following the generation of a potent gag SL9-specific CD8⁺ T cell response, suggesting either that the detected CD8⁺ T cell responses did not exert immune pressure on the SL9 epitope or that sequence variation within this epitope may impair viral fitness.
Figure 4.5

A.

<table>
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Figure 4.5: Overlapping gag peptides (15 a.a.) were pooled (A-V) and used to map HIV-specific CD8+ T cell responses in infant P-1115. (a) At week 6 of therapy, the gag-specific response is tightly focused on the A*0201-restricted VV9 epitope at the p24/p2 junction (peptide 91). (b) Conversely, by week 77 on therapy, the gag-specific response has dramatically shifted to the A*0201-restricted SL9 epitope in p17 (peptides 19 and 20). TNTC = too numerous to count.
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Table 4.4: Sequence analysis of the SL9 gag CD8⁺ T cell epitope from infant P-1115 reveals no deviation from the common SLYNTVATL epitope motif, despite a marked shift in CD8⁺ T cell epitope usage during this period.
G. Discussion.

In this chapter, HIV-1-specific CD8⁺ T cell responses were detected in 8 of 13 infants <6 mo of age, prior to initiation of ART. Responses against HIV-1 gag and nef were most commonly detected, but a significant portion of detectable responses were directed at other HIV-1 accessory proteins (tat, rev, vif, and vpr). These data suggest that the accessory proteins commonly serve as targets in the overall HIV-specific CD8⁺ T cell response during early vertical infection.

The disparate tat and rev-specific responses generated by infants P-1042 and P-1043 illustrate how HLA class I genotype can impact the generation of CD8⁺ T cell immunity. Furthermore, the identification of a novel and unique tat epitope highlights the challenge to the discovery of additional, perhaps sub-dominant CD8⁺ T cell epitopes that may, in aggregate, contribute to the overall HIV-specific response.

Finally, differential epitope recognition was observed over the course of HIV-1 infection in an infant. Viral sequence analysis at timepoints spanning this change in immune response failed to detect evidence of immune escape mutations within the SL9 epitope.
CHAPTER V

HIV-1 Replication Increases HIV-Specific CD4+ T Cell Frequencies but Limits Proliferative Capacity in Chronically-Infected Children.

A. Study population characteristics.

Peripheral whole blood samples were acquired from 44 children with established HIV-1 infection. Characteristics of the study cohort are presented in Table 5.1. Study children were placed into groups based on plasma HIV-1 load and ART history. Children in group A (n=16) were on combination ART and had plasma HIV-1 RNA <50 copies/mL. Children in group B (n=22) were also on combination ART but had plasma HIV-1 RNA >50 copies/mL (median: 1316 copies/mL; range: 99—57901 copies/mL). Age at study entry, median CD4+ T cell counts, and median duration of ART prior to study enrollment did not differ significantly between these two groups. Several children (group C, n=6) were either not receiving ART or were not adherent to a prescribed therapy (median plasma HIV-1 RNA: 60603 copies/mL; range: 11427—259350 copies/mL). Finally, 10 HIV-uninfected adults (group D) served as negative control donors.

B. HIV-1 gag p55-specific CD4+ T cells are commonly detected in children with established HIV-1 infection.
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<th>Plasma HIV-1 RNA (copies/mL)</th>
<th>Antiretroviral Therapy</th>
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<td>C</td>
<td>P-1017</td>
<td>13.8</td>
<td>+</td>
<td>6</td>
<td>122</td>
<td>100085</td>
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<tr>
<td>n=6</td>
<td>P-1046</td>
<td>6.4</td>
<td>-</td>
<td>28</td>
<td>736</td>
<td>259350</td>
<td>ZDV, 3TC, BMS</td>
<td>3.7</td>
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<tr>
<td></td>
<td>P-1107</td>
<td>13.4</td>
<td>+</td>
<td>35</td>
<td>502</td>
<td>11427</td>
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<td></td>
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<td>31</td>
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<td>21121</td>
<td>AZT, ddl, D4T, NVP, NFV</td>
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<td></td>
<td>P-1199</td>
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<td>-</td>
<td>24</td>
<td>299</td>
<td>13867</td>
<td>d4T, 3TC, BMS</td>
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<td></td>
<td>P-1325</td>
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<td>125755</td>
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<td>Median</td>
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<td>401</td>
<td>60603</td>
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</table>

Table 5.1: Study population characteristics.
Using an intracellular cytokine staining assay (ICS; Figure 5.1), HIV-1 p55-specific CD4+ T cell responses were detected in 38 (86%) of 44 study children at a median frequency of 0.34% (range: 0.02—2.88%) of CD4+/CD45RO+ small lymphocytes. No relationships between age, CD4+ T cell percentage or count and the detection or frequency of p55-specific CD4+ T cell responses were noted.

To confirm the HIV-1 specificity of the detectable CD4+ T cell IFN-γ responses, blood samples from 10 HIV-1-uninfected adult donors were tested (group D). HIV-1 p55-specific CD4+ T cell IFN-γ responses were not detected in the peripheral blood samples of any individuals in this group.

C. *HIV-1 gag* p55-specific CD4+ T cells are detected more commonly and at higher frequencies in children with ongoing HIV-1 replication.

Interestingly, 5 of the 6 children without detectable HIV-1 p55-specific CD4+ T cell responses also had plasma HIV-1 RNA <50 copies/mL. We therefore went on to examine the relationship between plasma HIV-1 load and HIV-1 p55-specific CD4+ T cells. HIV-1 p55-specific CD4+ T cell responses were less commonly detected in children with plasma HIV-1 RNA <50 copies/mL than in children with ongoing replication (Table 5.2). HIV-1 p55-specific CD4+ T cell responses were detected in 11 (69%) of 16 children with undetectable HIV-1 plasma loads while HIV-1 p55-specific CD4+ T cell responses were detected in 27 (96%) of 28 children with plasma HIV-1 RNA >50 copies/mL. In addition, higher frequencies of HIV-1 p55-specific CD4+ T cells were detected in children
Figure 5.1

- SEB
- CMV pp65
- HIV-1 p55
- No Antigen

P-1047 HIV+
P-008 HIV-
Figure 5.1: Detection of ex vivo HIV-1 gag p55-specific and CMV pp65-specific CD4+ T cell IFN-γ responses by intracellular cytokine staining. Shown is a representative flow cytometry profile of a child with detectable IFN-γ responses against both HIV-1 p55 and CMV pp65 protein antigens. After gating on small lymphocytes, CD4+/CD45RO+ double positive cells were analyzed for co-expression of IFN-γ and the activation marker CD69.
Table 5.2

<table>
<thead>
<tr>
<th>HIV-1 Replication Status</th>
<th>Proportion of Responders</th>
<th>p-value (Fisher’s Exact Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50 copies/mL</td>
<td>11/16 (69%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>&gt;50 copies/mL</td>
<td>27/28 (96%)</td>
<td>8/14 (57%)</td>
</tr>
</tbody>
</table>

**Table 5.2:** Forty four children infected with HIV-1 were analyzed for the presence of HIV-1 and CMV-specific CD4⁺ T cell IFN-γ responses. The proportion of children with plasma HIV-1 loads <50 copies/mL (undetectable; group A) or >50 copies/mL (groups B and C) with positive CD4⁺ T cells responses against HIV-1 p55 and CMV pp65 antigens are listed. Only children co-infected with CMV were considered in the analysis of CMV-specific responses. CMV-specific responses were not studied in all co-infected children due to insufficient specimen samples.
with active viral replication than in children with suppressed viral replication. As shown in Figure 5.2A, HIV-1 p55-specific CD4$^+$ T cell responses were detected at higher frequencies in group B (median: 0.57%; range: 0.08—1.80%) and in group C (median: 0.30%; range: 0.04—2.88%) than in group A (median: 0.14%; range: 0.02—0.7%). While the difference in HIV-1 p55-specific CD4$^+$ T cell frequencies between group A and group B was statistically significant (Wilcoxon Rank Sum: p=0.004), the difference in HIV-1 p55-specific CD4$^+$ T cell frequencies between groups A and C was not statistically significant. The latter may be due to alteration of the relationship between viral replication and CD4$^+$ T cell IFN-γ responses by CD4$^+$ T cell depletion, as discussed below.

**D. Cross-clade stimulation of HIV-1 gag p55-specific CD4$^+$ T cell responses.**

The use of a clade B-derived (SF2 isolate) p55 protein antigen raised the concern that HIV-1 p55-specific CD4$^+$ T cell responses may not be detected in children infected with other HIV-1 subspecies. Five children were born to women who acquired HIV-1 in areas in which non-clade B viruses predominate (P-1209 and P-1326, Zimbabwe; P-1210, South Africa; P-1211, Cambodia; P-1318, Ivory Coast). Responses were detected in 2 children (P-1210 and P-1326), born to mothers most likely infected with HIV-1 clade C viruses. Importantly, both responders had detectable plasma HIV-1 RNA, whereas each of 3 children lacking detectable p55-specific CD4$^+$ T cell responses presented with plasma HIV-1 RNA <50 copies/mL.
Figure 5.2

**Panel A**

HIV-1 p55 (gag)

- **A**: n=16
- **B**: n=22
- **C**: n=6
- **D**: n=10

p = 0.004

**Panel B**

CMV pp65 ( tegument)

- **A**: n=5
- **B**: n=10
- **C**: n=4
- **D**: n=2

p = 0.028
Figure 5.2: Frequency of ex vivo (a) HIV-1 gag p55-specific and (b) CMV pp65-specific CD4+ T cell IFN-γ responses in HIV-1 chronically-infected children. The percentage of CD4+/CD45RO+ small lymphocytes that produce IFN-γ and express CD69 in response to specific viral antigen stimulation is shown by patient group, as described in Table II (children with <15% CD4+ T cells (○); >15% CD4+ T cells (●)).
E. CMV pp65-specific CD4⁺ T cells are detected at higher frequencies in children with <50 copies/mL plasma HIV-1 load.

To further our understanding of virus-specific CD4⁺ T cells in HIV-infected children, CMV tegument pp65-specific CD4⁺ T cell frequencies were also measured in 19 HIV-1 and CMV co-infected children. CMV pp65-specific CD4⁺ T cell IFN-γ responses were detected in 13 (68%) of 19 children at a median frequency of 0.18% (range: 0—4.37%). As presented in Table 5.2, CMV-specific CD4⁺ T cell responses were detected more frequently in children with plasma HIV-1 RNA <50 copies/mL (5/5, 100%) than in those children with active HIV-1 replication (8/14, 57%). The difference in proportion of responders did not reach statistical significance, but suggested a trend towards an inverse relationship between active HIV-1 replication and the presence of CMV-specific CD4⁺ T cell responses. To further investigate this relationship, the frequencies of CMV pp65-specific CD4⁺ T cell IFN-γ responses were plotted by group. As illustrated in Figure 5.2B, all 5 children in group A had detectable pp65-specific CD4⁺ T cell responses at a median frequency of 1.07% (range: 0.1—4.37%). Conversely, only 5 (50%) of 10 children in group B had detectable pp65-specific CD4⁺ T cell IFN-γ responses (median: 0.07% range: 0—0.68%). These responses were significantly lower than those detected in group A (Wilcoxon Rank Sum: p=0.028). CMV pp65-specific CD4⁺ T cell IFN-γ responses were detected in 3 (75%) of 4 children in group C at intensities similar to those found in group B (median: 0.016%, range: 0.03—0.25%).
To confirm the specificity of the detectable CD4$^+$ T cell IFN-$\gamma$ responses, blood samples from 2 HIV-1 and CMV-uninfected adult donors were tested (group D). No CMV pp65-specific CD4$^+$ T cell IFN-$\gamma$ responses were detected in these individuals.

F. Low frequency virus-specific CD4$^+$ T cell IFN-$\gamma$ responses in children with depleted CD4$^+$ T cells.

CD4$^+$ T cell percentages of <15% are associated with higher mortality in HIV-1-infected children (51). Within the cohort, only 2 patients in group C, P-1017 and P-1325, presented with severely depressed CD4$^+$ T cell percentages (<15%) and counts (<150 cells/μL). Despite high-level viremia in both children (>10$^5$ copies/mL), HIV-1 p55-specific CD4$^+$ T cell IFN-$\gamma$ responses (Figure 5.2A, open circles) were either undetectable (P-1017) or low frequency (P-1325; 0.04%). Similarly, CMV-specific IFN-$\gamma$ responses (Figure 5.B, open circles) were either undetectable (P-1325) or low frequency (P-1017; 0.08%). These data suggest that CD4$^+$ T cell depletion may alter the previously observed relationship between plasma HIV-1 load and the intensity of p55-specific CD4$^+$ T cell IFN-$\gamma$ responses.

G. Ongoing HIV-1 replication is associated with detectable HIV-specific CD4$^+$ T cell responses.
Despite undetectable HIV-1 viral loads, some variability was observed in HIV-1 p55-specific CD4+ T cell IFN-γ responses in group A. HIV-1 2-LTR DNA circles were measured in the PBMC obtained from 14 children in group A at the same timepoints used in the ICS data presented above. Limited PBMC sample volumes precluded further study of the remaining 2 children (P-1033 and P-1035). Two-LTR DNA circles are abortive products of HIV-1 reverse transcription that occur when proviral DNA fails to integrate into the host genome (52). The presence of 2-LTR DNA circles within peripheral blood lymphocytes represents ongoing HIV-1 replication. Nine children in whom HIV-1 p55-specific CD4+ T cell IFN-γ responses (median frequency: 0.22%; range: 0.09—0.70%) were detected were defined as “responders,” As illustrated in Figure 5.3, HIV-1 2-LTR DNA circles were detected in all 9 responder children, at a median frequency of 3.5 copies/10^6 PBMC. By contrast, 2-LTR DNA circles were detected at a frequency of <1 copy/10^6 PBMC in all 5 non-responder children. The difference in the median frequencies of 2-LTR DNA circles between the two groups proved to be significant (Wilcoxon Rank Sum: p=0.04).

H. Changes in HIV-1 gag p55-specific CD4+ T cell frequencies are temporally associated with changes in plasma HIV-1 load.

Since our data suggested an association between HIV-1 replication and HIV-1 p55-specific CD4+ T cell IFN-γ responses, follow-up ICS assays were performed in several children who returned to the clinics. In most cases, plasma
Figure 5.3: HIV-1 2-LTR DNA circle copy numbers in children with plasma HIV-1 RNA <50 copies/mL and detectable ("responders") or undetectable ("non-responders") HIV-1 gag p55-specific IFN-γ responses.
HIV RNA loads and HIV-1 p55-specific CD4\(^+\) T cell frequencies were stable over time (data not shown). However, changes in plasma HIV-1 RNA levels were accompanied by changes in p55-specific CD4\(^+\) T cell frequencies (Figure 5.4). For example, patient P-1320 presented with plasma HIV-1 RNA of 24,514 copies/mL and an HIV-1 p55-specific CD4\(^+\) T cell IFN-\(\gamma\) response of 1.80% (Figure 5.4a). Following a change in therapy and a reduction of viral load by over 1 log, the p55-specific CD4\(^+\) T cell frequency fell to 0.57%. Conversely, child P-1207 presented with plasma HIV-1 RNA <50 copies/mL plasma HIV-1 load and a weak HIV-1 p55-specific CD4\(^+\) T cell IFN-\(\gamma\) response (0.09%; Figure 5.4b). Over the course of two months, HIV-1 RNA became detectable in this child’s plasma (~1100 copies/mL at two timepoints) and the frequency of HIV-1 p55-specific CD4\(^+\) T cell responses rose to 0.90%. The temporal association between plasma HIV-1 load and the frequency of p55-specific CD4\(^+\) T cell IFN-\(\gamma\) responses again illustrates the dependency of HIV-specific CD4\(^+\) T cell responses on the availability of HIV-1 antigen.


Nineteen children studied using the ICS assay (8 in group A, 9 in group B, 2 in group C) were concurrently tested for the presence of lymphoproliferative responses (LPA) against HIV-1 p55 (Figure 5.5); HIV-1 p55-specific IFN-\(\gamma\) secretion was detected in 16 (84%) of these 19 children. By contrast, HIV-1 p55-
specific lymphoproliferative responses were detected in only 6 of 19 (32%) children tested. LPA responses were detected in 4 of 8 (50%) children with <50 copies/mL plasma HIV-1 load (group A), but were detected in only 2 children (P-1012 in group B and P-1107 in group C) with plasma HIV-1 RNA >50 copies/mL. CD4⁺ T cell counts were similar in both groups, suggesting that the observed differences in HIV-1 p55-specific LPA responses were due to differences in viral load. HIV-1 p55-specific LPA responses were not detected in 6 HIV-uninfected donors (group D). Interestingly, there was no apparent relationship between the presence of p55-specific LPA responses and the frequency of CD4⁺ T cell IFN-γ responses (data not shown), although concurrent p55-specific CD4⁺ T cell IFN-γ responses (responder median: 0.68%; range: 0.13—0.86%). were detected in 5 of the 6 (83%) LPA responders. HIV-1 p55-specific CD4⁺ T cell IFN-γ responses were also detected in 11 of 13 (85%) LPA non-responders.
**Figure 5.4:** Temporal association of changes in HIV-1 gag p55-specific CD4⁺ T cell IFN-γ responses in children with changes in plasma HIV-1 RNA levels. Plasma HIV-1 load (□) and HIV-1 gag p55-specific CD4⁺ T cell IFN-γ frequencies (●) are plotted for patients P-1320 and P1207.
Figure 5.5: HIV-1 gag p55-specific CD4$^+$ T cell *in vitro* proliferation in children with suppressed or active HIV-1 replication. Stimulation indices greater than 3 were considered positive.
J. Discussion.

Data from the present study demonstrate that ex vivo HIV-1 and CMV-specific CD4⁺ T cell IFN-γ responses are commonly detected in children with established HIV-1 infection, despite the diverse therapeutic, clinical, and virological backgrounds of these children. Responses were more commonly detected and were detected at higher frequencies in the circulation of children with plasma HIV-1 RNA >50 copies/mL than in children with plasma HIV-1 RNA <50 copies/mL. Conversely, CMV pp65-specific CD4⁺ T cell IFN-γ responses were more commonly detected and were detected at higher frequencies in children with undetectable plasma HIV-1 load. These data suggest that CD4⁺ T cell IFN-γ responses can be primed and maintained in vertically-infected children. Furthermore, the detection of HIV-specific CD4⁺ T cells in most children, at frequencies comparable to those found in adults, indicates that the generation and maintenance of these cells are not appreciably altered by long-standing HIV-1 replication. However, the weak or undetectable HIV-1-specific CD4⁺ T cell responses detected in 2 children with severely depleted CD4⁺ T cell percentages suggests the importance of immune competence in maintaining these responses over time. Our findings are compatible with studies in chronically-infected adults that demonstrate that HIV-1 and other virus-specific CD4⁺ T cells are not deleted following prolonged viremia (41, 42, 44, 45).

In children with plasma HIV-1 RNA <50 copies/mL, HIV-1 p55-specific CD4⁺ T cell IFN-γ responses were detected only in the peripheral blood of children in
whom 2-LTR DNA circles were detected using a real-time PCR-based assay. While some groups have questioned the validity of 2-LTR circles as a marker of ongoing viral replication (53, 54), the correlation between the detection of HIV-1 p55-specific CD4⁺ T cell IFN-γ responses and the presence of 2-LTR DNA circles underscores their utility as a surrogate of covert viral replication. These data illustrate the ability of ongoing HIV-1 replication to drive and maintain HIV-specific CD4⁺ T cell IFN-γ responses, and that low-level HIV-1 replication, undetectable by ultrasensitive quantitation assays, is sufficient to maintain HIV-specific CD4⁺ T cell frequencies comparable to those found in children with significantly higher viral burdens.
A. Thesis Overview

A better understanding of the relationship between HIV-1 replication and HIV-specific immunity in vertically-infected infants and children is necessary for the successful development of effective treatment strategies, including a protective HIV-1 vaccine. Experimental data presented in this thesis dissertation examined the frequency, specificity, and timing of pediatric T cell responses against HIV-1.

As illustrated in chapter 3, HIV-1-specific CD8+ T cell responses were less commonly detected in younger infants (<6 months) than in older infants (>6 months) prior to ART. Following the initiation of ART, HIV-1-specific CD8+ T cell responses were uncommonly detected in infants with persistent control of viral replication. The detection of CMV-specific CD8+ T cell responses in young HIV-1/CMV coinfected infants indicates that young infants are capable of generating and maintaining CD8+ T cell response directed against a vertically-acquired viral infection.

In chapter 4, HIV-specific CD8+ T cell responses were detected more frequently using an expanded panel of HIV-1 antigens. CD8+ T cell IFN-γ responses against HIV-1 accessory gene products represented nearly half of detectable responses. This finding suggests that inclusion of HIV-1 accessory gene products into vaccine strategies may improve immunogenicity and efficacy.
A detailed analysis of early HIV-specific CD8$^+$ T cell responses in a pair of fraternal twins underlines the challenges to generating broad and intense CD8$^+$ T cell responses in a diverse population. Finally, the potent gag-specific CD8$^+$ T cell response in a young infant is characterized. Despite strong gag-specific CD8$^+$ T cell responses during the first 2 years of life, a marked shift in gag epitope usage is observed. Viral sequence analysis revealed that epitope escape, or the evolution of viral species with sequence mutation that would inhibit CD8$^+$ T cell recognition, does not mediate the epitope shift observed in this infant.

Finally, in chapter 5, HIV-specific CD4$^+$ T cell IFN-γ responses were commonly detected in a large cohort of children with established HIV-1 infection. A close relationship between the presence and frequency of HIV-specific CD4$^+$ T cells and plasma HIV-1 load was observed. The majority of children with active HIV-1 replication failed to mount appreciable HIV-specific proliferative responses, despite displaying high frequencies of IFN-γ producing cells. These data support the uninterrupted use of combination ART in HIV-1-infected children to preserve HIV-1 and other virus-specific CD4$^+$ T cell function by controlling HIV-1 replication. Vaccination to stimulate HIV-specific CD4$^+$ T cell responses under continued ART may improve the long-term control of viral replication, and perhaps allow the eventual discontinuation of ART.

B. Rationale for the study of pediatric HIV-specific T cell immunity.
Antiretroviral therapy (ART) has proven to be an important tool in the prevention of mother-to-child transmission of HIV-1 infection, but it is clear that a long-term prevention and treatment strategy can not rely on ART alone. Early treatment of HIV-infected infants with ART effectively reduces HIV-1 replication and has dramatically improved the long-term prognosis for infants and children living with HIV-1. Single or short-course ART may hold promise in regions of the developing world, but the expense and expertise required to dispense this care has limited its impact. Even with reduced perinatal transmission rates, current ART interventions do not appear to provide protection against HIV-1 transmission through breastmilk. Finally, the lack of HIV-1 antigens for presentation to HIV-specific CD8+ T cells in treated infants and children may not allow eventual cessation of antiretroviral therapy. A neonatal vaccine to boost HIV-specific cellular immune responses in infants may provide protective immunity to HIV-1, or at least reduce HIV-1 viral burden and delay immunodeficiency disease progression. A better understanding of HIV-specific CD8+ and CD4+ T cell responses in infants and children is vital to the development of an effective pediatric vaccine.

C. HIV-specific CD8+ T cell responses during infancy.

Data from adult human (21, 55, 56) and simian (31, 32) studies have demonstrated the importance of the HIV-1-specific CD8+ T cell response in controlling primary HIV-1 viremia. The expansion of HIV-1-specific CD8+ T cell
populations during the acute phase of infection is thought to contribute to the establishment of an equilibrium between viral replication and the host immune system (viral setpoint) that is predictive of subsequent disease progression. HIV-1-specific CD8$^+$ T cells also appear to play a critical role in the control of viral replication during the course of chronic infection (57).

While much effort has been made to characterize the breadth, intensity, and timing of HIV-1-specific CD8$^+$ T cell responses in infected adults, the generation and maintenance of HIV-1-specific CD8$^+$ T cells in vertically-infected infants are less well understood. A general paucity of PBMC samples and infrequent detection of antigen-specific CD8$^+$ T cells has made study of HIV-1-specific CD8$^+$ T cell responses difficult in young infants. Responses to reported immunodominant epitopes commonly recognized by adults with established infection (58) are rarely detected in young infants (our unpublished data). This may indicate differential recognition of HIV-1 epitopes over the course of infection (59) or that different HIV-1 peptide epitopes are preferentially recognized by young infants.

In chapter 3, the difference in detectable HIV-1-specific CD8$^+$ T cell responses between infants of the two age groups prior to therapy was highly significant and may reflect age-related differences in the dynamics of activation and expansion of antigen-specific CD8$^+$ T cells in young infants. In this regard, it is interesting to note that the infrequent detection of HIV-1-specific CD8$^+$ T cell responses in young infants prior to ART contrasts with the frequent detection of
HIV-1-specific CD8$^+$ T cell responses prior to therapy in adults with primary HIV-1 infection (33). HIV-1-specific CD8$^+$ T cell responses were lower in frequency and less broad in adults who initiated ART within 6 months of infection than in individuals who initiated ART >6 months following infection, suggesting that a reduction in viral replication during primary infection decreases the frequency and breadth of HIV-1-specific CD8$^+$ T cell responses. However, HIV-1-specific CD8$^+$ T cell responses were persistently detected in the majority of adults at least one year following the initiation of potent combination ART.

The more frequent detection of HIV-specific CD8$^+$ T cell responses (in 8 of 13 infants presented in chapter 4) stands in contrast to the data presented in chapter 3. Remarkably, the infants in both cohorts were quite similar with respect to age, standard of care, HIV-1 replication, and CD4$^+$ T cell counts. Moreover, the methodology used to detect responses (B-LCL/ELISPOT) was identical between studies, aside from the inclusion of recombinant tat, rev, vif, and vpr antigens. Considering the contribution of CD8$^+$ T cell responses specific for the HIV-1 accessory gene products to the overall anti-HIV response in the current study, the experimental results presented in chapter 3 most likely underestimated the level of HIV-specific CD8$^+$ T cell immunity present in the young infants.

Interestingly, HIV-specific CD8$^+$ T cell responses were only detected in 10 of 26 infants <6 mo of age if the results of both chapters are combined. Several unique characteristics of the neonatal cellular immune system and the dynamics of vertical HIV-1 infection may help to explain the infrequent detection of CD8$^+$ T
cell responses to HIV-1. First, the cellular immune system in neonates and young infants may have different requirements for the activation and expansion of antigen-specific CD8⁺ T cells. The antigen-processing and presentation capability of infant antigen presenting cells has been questioned, especially with regard to dendritic cell function (60). Inefficient presentation of HIV-1 antigens to naïve CD8⁺ T cells may hinder the generation of effector and memory CD8⁺ T cell populations. Second, Selin et al. have demonstrated that sequential viral infections shape the memory T cell pool through the expansion or deletion of cross-reactive CD8⁺ T cell populations (61, 62). Limited exposure to heterologous viruses during the first months of life may impair the generation and expansion of CD8⁺ T cell populations cross-reactive with HIV-1.

The detection of CMV-specific CD8⁺ T cells in 5 young HIV-1/CMV-coinfected infants (again, combining results of chapters 3 and 4) suggests that young infants are capable of generating virus-specific CD8⁺ T cell responses. CMV-specific responses were detected at all timepoints studied in 5 young coinfected infants, at frequencies similar to those detected in CMV seropositive, HIV-1-uninfected adults (unpublished data). Initiation of ART did not appear to alter CMV-specific CD8⁺ T cell frequencies over time. The detection of CMV-specific CD8⁺ T cells in 3 young HIV-1/CMV-coinfected infants suggests that young infants are capable of generating virus-specific CD8⁺ T cell responses and that the paucity of detectable HIV-1-specific CD8⁺ T cell responses represents a selective defect in the generation or maintenance of HIV-1-specific CD8⁺ T cells.
There are several potential explanations for the apparent selective defect in the generation or maintenance of HIV-1-specific CD8⁺ T cells in these young infants. First, murine models suggest that the development of neonatal CD8⁺ T cell responses is highly influenced by antigen load and the antigen presenting cell (63, 64). Differences in the kinetics and sites of HIV-1 and CMV replication may therefore lead to differential generation or maintenance of cellular immune responses directed against these viruses in coinfected hosts. Second, the acquisition of HIV-1 infection in the presence of high titers of passively-acquired maternal antibodies may also affect the generation of HIV-1-specific CD8⁺ T cell responses. The deletion of HIV-1-specific CD4⁺ T cells by the cytopathic effects of HIV-1 may contribute to the low frequency of HIV-1-specific CD8⁺ T cells. HIV-1-specific CD4⁺ T cell responses appear to be important for the generation and maintenance of potent HIV-1-specific CD8⁺ T cell responses (34, 39). For instance, several HLA class I alleles have been shown to correlate with more rapid HIV-1 progression, implying these alleles do not mediate numerous or potent HIV-specific CD8⁺ T cell responses (65). Viral polymorphisms or escape mutations passed on from the mother may inhibit the generation of detectable CD8⁺ T cell responses in the infant (66). Finally, the use of IFN-γ as the readout for antigen specificity may underestimate or completely miss populations of HIV-1-specific CD8⁺ T cells with alternate effector phenotypes.

D. Importance of HIV-1 accessory proteins in the CD8⁺ T cell response.
Although several CD8+ T cell epitopes have been identified within HIV-1 accessory proteins, they are few in number compared to those identified within the larger, canonical proteins gag, pol, and env. While it is tempting to conclude that HIV-1 accessory proteins are preferentially targeted during pediatric infection, it is more likely that the methodologies and reagents used in previous studies did not facilitate detection of accessory gene-specific responses. To illustrate, many investigators examining CD8+ T cell responses in adults (and infants/children) tend to focus on responses directed against epitopes within gag, pol, and env, largely because these antigens (peptides, whole proteins, recombinant vectors) are more readily available for experimental use. Additionally, many studies have relied heavily on HLA class I tetramers against “immunodominant” epitopes, found primarily within gag, to gauge the overall CD8+ T cell immune response against HIV-1. Reliance on HLA class I tetramers, typically manufactured using a limited set of class I A or B alleles, does not allow for a complete evaluation of all antigen-specific CD8+ T cells that may be present.

E. Role of HLA and viral sequence in shaping early CD8+ T cell responses.

The strikingly distinct CD8+ T cell responses detected in fraternal twins P-1042 and P-1042 underlines the challenges faced in selecting HIV-1 antigens appropriate for incorporation into a pediatric vaccine. The tat and rev-specific CD8+ T cell IFN-γ responses generated by twins P-1042 and P-1043,
respectively, were in the context of relatively obscure HLA class C alleles. Since the binding characteristics of many C loci remain unknown, currently available epitope prediction software tends to skew epitope mapping studies towards the A and B class I loci. Clearly, HLA class I genotype plays an important role in mediating the CD8⁺ T cell response, but uncommon viral polymorphisms within infected infants and children also make prediction and identification of CD8⁺ T cell epitopes difficult. The sequence of the novel tat-specific CD8⁺ T cell epitope recognized by P-1042 (restricted by HLA Cw*0303) is unique among published sequences in the Los Alamos Sequence Database (50). The use of HIV-1 antigens based on common lab strain clade B isolates may have failed to detect this tat-specific response. The parallel is clear—vaccine strategies based on consensus or lab-adapted isolates may fail to provide protective immunity.

F. CD8⁺ T cell responses and viral escape in HIV-infected infants.

Escape mutations within HIV-1 and SIV (in animal models) CD8⁺ T cell epitopes have been characterized in several studies (67, 68). The high error rate of the HIV-1 RT enzyme allows for the generation of viral variants that, in the face of selective pressures, generate non-synonymous base pair mutations within regions of the viral genome that benefit overall replicative "fitness." We hypothesized that the absence of SL9-specific CD8⁺ T cells during the first year of life in infant P-1115 was due to the transmission and outgrowth of viral species containing escape mutations within the SL9 epitope. One study has documented
the transmission of such immune escape mutations in human vertical HIV-1 infection, but the conserved nature of the SL9 epitope may not make it an attractive epitope for continued studies of transmittable mutations (66).

Interestingly, Goulder et al. have also described a group of HLA A*02 adults during acute and chronic HIV-1 infection with very similar shifts in epitope usage (59). Despite the frequent detection of gag SL9-specific CD8+ T cell responses in chronically-infected HLA A*02+ adults, no such responses or cell populations were detected in 11 adults during acute infection. Gag SL9-specific CD8+ T cells later became detectable in 2 subjects that were followed longitudinally. Additionally, analysis of viral sequences during acute viremia did not reveal substantial deviations from the standard SLYNTVATL sequence. The striking similarity of the late emergence of SL9-specific CD8+ T cells in the absence of viral escape variants between these adults and infant P-1115 (in our study) illustrates a common pattern for the establishment of CD8+ T cell dominance. This pattern appears to be unaffected by differences in age, and closely related to the resolution of primary viremia (mirrored in our infant by lowered HIV-1 replication from ART) and establishment of chronic infection.

G. HIV-specific CD4+ T cells and relationship with HIV-1 replication.

The past five years have seen marked progress in the study of HIV-specific CD4+ T cell populations, and how the frequency and function of these cells relate to HIV-1 replication. A cross-sectional study of HIV-1 p24-specific CD4+ T cell
proliferative responses found an inverse relationship between the presence of proliferative responses and plasma HIV-1 RNA in therapy-naïve, chronically-infected adults (39). The absence of HIV-specific CD4⁺ T cell proliferation in viremic individuals suggested the loss or dysfunction of these cells as a consequence of concurrent HIV-1 replication. Later, using a flow-based intracellular cytokine staining assay, HIV-specific CD4⁺ T cells producing IFN-γ were detected ex vivo in adults with progressive HIV-1 infection (41). HIV-1 gag-specific CD4⁺ T cells were more commonly detected and were detected at higher frequencies in HIV-1 progressors than in individuals with undetectable plasma HIV-1 loads, although no significant relationship between plasma HIV-1 RNA and the frequency of HIV-specific CD4⁺ T cell responses was observed. These data demonstrated that HIV-specific CD4⁺ T cells are not depleted from the circulation of individuals with long-standing HIV-1 replication. Recently, at least 2 groups have described the detection of IFN-γ-producing HIV-specific CD4⁺ T cells but diminished in vitro proliferative responses in individuals with ongoing HIV-1 replication, clarifying the seemingly discordant previous findings (44, 45). In long-term non-progressors (LTNP) with controlled HIV-1 replication, HIV-specific CD4⁺ T cell IFN-γ responses were found concurrently with HIV-1 proliferative responses. The lack of proliferative responses in adults with progressive HIV-1 infection suggested a specific functional defect in HIV-specific CD4⁺ T cells.

Available data suggest the selective impairment of HIV-specific T cell responses in early vertical infection. We have previously demonstrated the
delayed generation of HIV-specific CD8+ T cells in young infants (47, 49) and that HIV-specific CD4+ and CD8+ T cell responses are infrequently detected in the circulation of children who receive potent combination ART in the first few months of life (27). Until recently, however, most children did not receive early suppressive ART and persistently high plasma HIV-1 loads have been documented over the first several years of life (24). Robust viral replication in the setting of a developing immune system may dramatically alter the establishment and maintenance of HIV-specific CD4+ and CD8+ T cell effector and memory cell populations.

Most prior studies have not documented a strong relationship between CD4+ T cell responses and viral load (41, 42, 44, 45, 69). Pitcher et al. documented a difference in the frequency of HIV-specific CD4+ T cell IFN-γ responses between adults with active replication and those with long-term suppressed HIV-1 replication, but this difference was not statistically significant (41). We feel there are several reasons why such a relationship was not observed in previous reports. First, several studies focused on HIV-1-infected long-term non-progressors and compared the frequency of HIV-specific CD4+ T cells in these individuals to those found in active HIV-1 progressors (42, 44, 69). Several analyses have presented evidence of ongoing HIV-1 replication in LTNP despite undetectable plasma HIV-1 loads by standard quantitation assays (57, 70). The study of LTNP individuals may have minimized the differences in observed HIV-specific CD4+ T cell frequencies when compared to individuals with progressive
infection. Secondly, some studies used routine (limit of detection = 400 RNA copies/mL) rather than ultrasensitive (limit of detection = 50 RNA copies/mL) assays for the quantitation of plasma HIV-1 RNA, thereby making it likely that low levels of HIV-1 replication were not detected (41, 42, 45). Finally, not all plasma HIV-1 RNA is replication competent and it could be that HIV-specific CD4+ T cell frequencies correlate better with measures of recent replication events. Future studies that utilize high-resolution analysis of recent replication events or plasma HIV-1 load may continue to clarify the relationship between ongoing HIV-1 replication and the frequency of HIV-specific CD4+ T cells IFN-γ responses.

Aside from LTNP individuals, many studies have described weak or absent HIV-specific CD4+ T cell proliferative responses in viremic adults who did not receive ART during acute infection (39, 40, 44, 71). By contrast, the present study detected HIV-specific CD4+ T cell proliferative responses in children who did not receive ART during acute HIV-1 infection. Responses were primarily detected in children with plasma HIV-1 RNA <50 copies/mL. These data suggest that the major factor associated with reduced in vitro proliferative capacity is the presence of vigorous ongoing viral replication and that control of viral replication may allow the reversal of the proliferative defect. In this regard, the limited proliferative capacity of HIV-specific CD4+ T cells may be similar to those described in other virus-specific CD4+ T cell responses during acute infection (72).
There are several potential mechanisms to explain diminished in vitro p55-specific CD4+ T cell proliferation in children with ongoing HIV-1 replication. The recent demonstration by Douek et al. that HIV-specific CD4+ T cells are preferentially infected by HIV-1 raises the possibility that the absence of proliferative responses in viremic individuals may be a direct consequence of HIV-1 infection (73). Sieg and colleagues have reported that diminished CD4+ T cell proliferation in HIV-infected individuals is associated with reduced expression of several cell cycle proteins, resulting in early G₁ arrest (74). Observed imbalances in T cell nucleoside pools in HIV-1-infected individuals may inhibit normal cellular proliferation (75). Finally, continuous exposure of HIV-specific CD4+ T cells in chronically-viremic persons may allow "clonal exhaustion" that preclude the ability of these cells to proliferate in vitro and possibly, in vivo.

H. HIV-specific T cell immunity and HIV-1 replication

The data presented in this dissertation have detected and characterized both CD8+ and CD4+ T cell responses in pediatric subjects. The dependence of both arms of the T cell immune response on active HIV-1 replication to drive and maintain effector populations supports the use of HIV-1 antigens to immunize infants and children. It is abundantly clear, however, that traditional vaccination methods must be used to boost HIV-specific immunity. Structured therapy interruptions have been explored as a means of "auto-vaccinating" an individual to boost HIV-specific immune responses. While the merits of these treatments
are debatable, the loss of CD4\(^+\) T cell proliferative capacity in viremic individuals may not allow for the establishment of long-lived CD4\(^+\) and CD8\(^+\) T cell immunity. Furthermore, the preservation of CMV-specific CD4\(^+\) T cell populations in children with suppressed HIV-1 replication supports the use of combination ART when possible.

I. Conclusion.

The experiments detailed in this thesis dissertation have contributed to a more complete understanding of the timing, intensity, and breadth of pediatric T cell responses targeted against HIV-1. Specifically, the frequency and hierarchy of HIV-1-specific CD8\(^+\) T cells in vertically-infected infants were defined. Significant relationships between the presence, breadth, and intensity of HIV-specific CD8\(^+\) T cell responses and age and HIV-1 replication were noted. Furthermore, these data indicate that the HIV-1 accessory proteins play an important role in the pediatric CD8\(^+\) T cell response against HIV-1, and vaccine strategies may benefit from the inclusion of these immunogenic proteins. The role of epitope escape in pediatric infection was explored in a single infant with a dramatic shift in epitope usage during the first 2 years of life. The lack of escape mutation within epitope sequences suggests that alternative mechanisms, such as antigen processing or regulation of viral protein expression, may influence the specificity and timing of early HIV-specific CD8\(^+\) T cell responses. Finally, HIV-1-specific CD4\(^+\) T cell responses were studied in a large cohort of vertically-
infected children. The results of this study reveal a relationship between HIV-1 replication and the presence of HIV-1-specific CD4+ T cells, a relationship not reported in any previous study, pediatric or adult. The lack of CMV-specific CD4+ T cell IFN-γ responses, as well as HIV-specific CD4+ proliferative responses in children with progressive HIV-1 infection reaffirms the benefit of antiretroviral therapy and argues against therapy interruption schemes that may deteriorate antigen-specific CD4+ T cell responses.

In conclusion, data presented in this dissertation illustrate that HIV-specific T cell immune responses may be detected in vertically-infected infants and children. Vaccine strategies to prime and/or boost HIV-specific cellular immunity in vertically-infected infants and children may allow for continued control of HIV-1 replication through current therapy strategies and perhaps offer the hope that therapy may be removed as effective HIV-specific cellular responses are mounted.
References


