Fate of the HIV-specific immune response starting in primary infection

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fulfillment of the requirements of the degree of

Doctor of Philosophy

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Thesis Abstract

Two decades into the epidemic caused by the human immunodeficiency virus (HIV) and this virus has infected nearly 1% of the world's population. Highly active antiretroviral therapy (HAART) has transformed the epidemic from a death sentence into a chronic infection. However restricted access, complicated regimens, drug resistance, and long-term toxicities are all impediments to life long therapy for all those infected. These factors stress the need for vaccines and immunotherapeutic strategies that may be used in conjunction with antiretroviral drugs for the optimization of patient care.

It is clear that cytotoxic T lymphocytes are potent suppressors of viral replication throughout the clinical course of HIV infection. The research presented in this thesis focuses on the characterization of immune responses at different stages of HIV infection in order to gain a better understanding of the pattern of changes that occur within the host response to virus on and off therapy following infection.

The HIV specific immune response broadens during the first two months of infection, suggesting that while it is induced early during acute infection, the antiviral response evolves to respond to the virus. HIV specific immune responses can be preserved if HAART is initiated while the subject is in early PI, and appears to depend on the preservation of HIV specific CD4+ T cell help. Subjects remaining untreated in PI experience significant perturbations (expansions and contractions) in responses to individual HIV peptides over a 1 year period, which is characterized by an overall contraction in the breadth and persistence in the magnitude of their antiviral effector activity.

New strategies that map the specificity, breadth and magnitude of the HIV specific immune response testing all HIV expressed gene products were evaluated. The novel approach will strengthen conclusions derived from the types of studies conducted for this thesis by detecting all reactivities present in subjects being tested in an unbiased manner. Interferon- γ (IFN-γ) secretion in response to epitopes previously unknown to be immunogenic are frequently detected using this screening method in subjects in the chronic phase of infection. Use of this technique demonstrates that HAART-naive HIV+ chronically infected subjects in asymptomatic disease target Gag predominantly and possess variable breadths and magnitudes of responses to HIV.

Taken together, the data presented in this thesis have improved our understanding of the pattern of the evolution of the immune response over time. Data presented here have implications for HIV infected patient management, by providing new information useful for deciding whether the immunological benefit of initiating HAART early in infection outweighs toxicities associated with long term treatment. Finally, new strategies for the detection of HIV specific immune responses will further our ability to characterize the global HIV specific immune responses at all stages of HIV disease.

Resumé de la Thèse

Vingt ans c'est écoulé depuis l'apparition du virus de l'immunodéficience acquise. Depuis, ce virus a réussi d'infecter presque 1% de la population mondiale. Heureusement, le traitement antiretroviral (TAR) a donné espoir aux patients et à la communauté scientifique en transformant cette maladie mortelle en une condition chronique. Grâce à cette dernière, les personnes infectées ont une espérance et une qualité de vie grandement améliorée. Toutefois, la disponibilité des médicaments, l'adhésion au régime, la résistance virale et la toxicité systémique sont d'importants facteurs à prendre en considération lors de l'administration à long terme de le TAR. Ces complications mettent d'autant plus l'emphase sur l'urgence du développement de vaccins ou autre interventions thérapeutique afin de mieux gérer la qualité de vie du patient.

Au cours d'une infection avec le VIH, il a été démontré que les lymphocytes cytotoxiques aide à contrôler la charge virale. La recherche présentée dans cette thèse à pour but de caractériser la réponse immunitaire à différents stages de la maladie afin de mieux comprendre le délicat équilibre entre l'hôte et le virus. De plus cette recherche vise à comprendre les changements dans la réponse immunitaire lors du traitement.

La réponse immunitaire au VIH se diversifie durant les 2 premiers mois de l'infection indiquant l'évolution de la réponse antivirale avec le temps. Ces réponses immunitaires spécifiques au VIH peuvent être conservées si le TAR est initiée lors de la période d'infection primaire. Il semblerait que ces réponses dépendent du maintient des lymphocytes CD4+ spécifique au virus. Durant la période d'infection primaire, les patients n'ayant pas été traités, présentent des anomalies de nature qualitatives aux niveaux des peptides HIV-specifiques reconnus par leur système immunitaire. Ces

anomalies se présentent durant la première année d'infection sous la forme d'une diminution globale des épitopes HIV-spécifiques reconnues par le patient tout en maintenant l'intensité des réponses spécifiques.

Des nouvelles stratégies telle que le matrix ELISPOT sont disponibles aujourd'hui. Ces dernières nous permettent de cartographier la spécificité, la diversité et l'intensité des réponses immunitaires spécifique à toutes les protéines virale exprimée sans prendre en considération le HLA du patient. De plus, de nouveaux épitopes non caractérisé sont fréquemment détectés chez des patients en infection chronique. Cette méthode a également démontré que des patients en infection non traités durant la période asymptomatique (chronique) de la maladie cible majoritairement la région virale Gag avec une diversité et intensité variable.

En conclusion, les données présentées dans cette thèse contribuent à mieux comprendre l'évolution de la réponse immunitaire dans le temps. Cette étude procure également de l'information quant à l'initiation du traitement et ses effets bénéfiques sur le système immunitaire versus le risque d'un traitement prolongé avec ses effets secondaires toxiques. Finalement de nouvelle stratégie pour la détection des réponses immunitaires spécifiques globales contre le VIH permettent de mieux caractériser le statut immunitaire d'un patient durant toutes les phases de la maladie.

Acknowledgements:

I would like to begin by thanking my supervisor, Dr. Nicole Bernard, for her superb mentorship, guidance, and friendship. Dr. Bernard, you have helped make research a life-long passion that I will cherish forever. I want to thank you for your steadfast support and your never ending patience.

I would like to thank my co-supervisor, Dr. Chris Tsoukas, for his mentorship, enthusiasm for research, and for his undying support. I feel that you have given me the drive to commit my life to the pursuit of knowledge.

Additionally, I would like to thank my colleagues and lab-mates (past and present), particularly Famane Chung and George Makedonas, who stood by me throughout the process. I mostly want to thank you all for your friendship, but also for your support, your help, and your inspiring thoughts. You have all truly made this process a pleasure.

I am grateful for the advice and support I received from my advisor, Dr. Danuta Radzioch, and my thesis committee member, Dr. Brian Ward.

Finally, I would like to thank my family, whose support means the world to me. Your words of encouragement have helped me more than you can imagine.

Preface

The studies which are described in the thesis have been carried out by the author of the thesis under the guidance and supervision of Dr. Nicole Bernard at the Montreal General Hospital.

> Chapter 2: ***HIV-specific effector CD8+ T cell activity in patients in primary HIV infection***. Galit Alter, Alefia Merchant, Christos M. Tsoukas, Danielle Rouleau, Roger P. LeBlanc, Pierre Côté, Jean-Guy Baril, Réjean Thomas, Vinh-Kim Nguyen, Rafik-Pierre Sékaly, Jean-Pierre Routy, Nicole F. Bernard
> J. Infect. Dis. 2002 185(6):755-765.

The project was initially conceived by Dr. Nicole Bernard. The author of this thesis is the first author on this paper and is directly responsible for the experimental work on the primary infection subjects described in the study. Additional help was provided by Alefia Merchant, who performed data collection on the chronically infected subjects. Patients were followed at various clinics, by physicians that are all included as co-authors on the study. Additionally, the majority of the primary infection samples were processed and delivered by Dr. Rafick-Pierre Sekaly and his team. The remaining PI samples and all chronically infected samples were provided by Dr. Chris Tsoukas. The database for the Quebec Primary Infection Cohort was maintained by Mr. Mario Legault.

> Chapter 3*: **Longitudinal assessment of changes in HIV-specific effector activity in HIV infected patients starting HAART in primary infection.*** Galit Alter, George Hatzakis, Christos Micheal Tsoukas, Karen Pelley[*], Danielle Rouleau, Roger LeBlanc, Jean-Guy Baril, Harold Dion, Eric Lefebvre, Réjean Thomas[§], Pierre Côté, Normand Lapointe, Jean-Pierre Routy, Rafik-Pierre Sékaly[|], Brian Conway, Nicole Flore Bernard.
> J. Immunol. 2003 171(1):477-488.

The project was designed by Galit Alter and Dr. Nicole Bernard. The author of the thesis is the first author and is directly responsible for all experimental work and data analysis. Dr. George Hatzakis was vital in the statistical analysis presented in this paper. As

mentioned for chapter 2, all physicians whose subjects were included in this study are listed as co-authors. Dr. Rafick-Pierre Sekaly provided the majority of the primary infection samples. The remaining PI samples and all chronically infected samples were all supplied by Dr. Christos Tsoukas. Dr. E. Delwart provided the study with all detuned assay data. All the clinical data was compiled and maintained in a database for the Quebec Primary Infection Cohort by Mr. Mario Legault.

> Chapter 4: ***Assessment of longitudinal changes in HIV-specific effector activity in subjects undergoing untreated primary HIV infection***. Galit Alter, Christos Micheal Tsoukas, Danielle Rouleau, Pierre Côté, Jean-Pierre Routy, Rafik-Pierre Sékaly[1], Nicole Flore Bernard.
>
> Submitted to Blood August 16, 2003.

The study design, experimental work, and data analysis was performed by the author of this thesis, first author on the paper. The idea was conceptualized by Galit Alter and Dr. Nicole Bernard. Dr. Hugo Soudeyns provided helpful criticism that was important during manuscript editing. Again, all physicians whose subjects were included in this study are listed as co-authors, Mr. Mario Legault maintained the clinical databse, and Dr. Rafick-Pierre Sekaly provided the primary infection samples.

> Chapter 5: ***Evaluation of the matrix ELISPOT.*** Galit Alter, Marie-Pierre Boisvert, Jean-Pierre Routy, Nicole F. Bernard.

The technical approach described in chapter 5 was initially conceived by Dr. Nicole Bernard. The author of this thesis was responsible for all analysis, study design and data collection in order to evaluate this novel technique. The experimental work was performed by the author of this thesis, who is also the first author on this paper, with the help of Marie-Pierre Boisvert. Samples were provided by Dr. Jean-Pierre Routy.

Chapter 6: ***Characterization of the breadth, magnitude, and specificity of the HIV-specific effector response in 24 HLA-A2 Untreated chronically infected subjects.*** Galit Alter, Yoav Peretz, Marie-Pierre Boisvert, Christos M. Tsoukas, Nicole F. Bernard.

The study described in chapter 6 was conceived by the author of this thesis, the first author on the paper. The data collection was performed by Galit Alter with the help of Yoav Peretz. CTL assays were performed by Galit Alter in collaboration with Marie-Pierre Boisvert. All analyses were performed by Galit Alter. All samples were provided by Dr. Chris Tsoukas.

Abbreviations

ADCC	antibody-dependent cell mediated cytolysis
AIDS	acquired immunodeficiency syndrome
AIED	acute infection/early disease
APC	antigen presenting cell
ARV	AIDS associated retrovirus
CAF	CD8+ T cell antiviral factor
CDC	center of disease control
cdk	cyclin-dependent kinase
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EBV	Epstein Barr virus
Env	envelope
EU	exposed-uninfected
FDC	follicular dendritic cell
GRID	gay-related immunodeficiency disease
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV	human T cell leukemia virus
IFN-γ	interferon- γ

Ig	immunoglobulin
IN	integrase
KS	Kaposi's sarcoma
LAS	lymphadenopathy syndrome
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LTNP	long-term non-progressor
LTR	long-terminal repeats
MA	matrix (p17)
MHC	major histocompatibility complex
MVA	modified-vaccinia virus Ankara
Nef	negative factor
NK	natural killer cell
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NSI	non-syncitium inducing
PBMC	peripheral blood mononuclear cell
PCP	pneumocystis carinii pneumonia
PI	primary infection
PIC	pre-integration complex
PR	protease
RT	reverse transcriptase
RTC	reverse transcriptase complex

SI	syncitium inducing
SIV	simian immunodeficiency virus
STI	structured treatment interruption
TAP	transporters associated with antigen processing
Th	CD4+ T helper cell
TCR	T cell receptor
TNF-α	tumor necrosis factor- α
TREC	T cell receptor excision circle
tRNA	transfer RNA
Vif	viral infectivity factor
Vpr	viral protein R

Chapter 1: Introduction

1.1 Discovery of the Human Immunodeficiency Virus:

In 1980, Dr. Micheal Gottlieb was alarmed by a case of a 33-year-old man he had recently examined. He wrote that the patient was: "pale, almost ashen; extremely thin, bordering on anorexia; a mouth full of cottage cheese, indicative of a fungal infection; coughing uncontrollably, and evincing severe lung pain"[1](p293). This young, otherwise healthy patient was experiencing a rare form of pneumonia, mainly diagnosed in transplant patients and the elderly called *Pneumocystis carinii* pneumonia (PCP). The patient had near to normal antibody responses, but had virtually no helper (CD4+) T cells. Dr. Gottlieb was in shock as he watched the patient's body fail one organ at a time over a short period. Meanwhile, Dr. Joel Weisman, treating a sizeable gay male population, began to take note of a group of patients complaining of fatigue[1]. By 1981, Dr. Weisman began to document significant changes in this particular group of men. They exhibited swollen lymph nodes, major weight loss, and the same rare pneumonia seen in Dr. Gottlieb's patient, i.e. PCP. During this period, the Center of Disease Control (CDC) noted a dramatic 4-fold increase in the number of prescriptions for anti-PCP drugs[1]. By 1981, the CDC published a report linking Kaposi's sarcoma (KS), a cancer that was relatively rare in the United States, with PCP and homosexuality in New York City and San Francisco. By the end of the year, the CDC documented a group of 107 cases of KS and/or, PCP[1]. A study conducted around the same time reported a strong association between the appearance of KS or PCP and promiscuity within the gay population. The illness was therefore named Gay Related Immunodeficiency Disease (GRID)[1].

Dr. Auerbach, a CDC official, began the difficult task of tracing the origins of the epidemic in the US[1]. Dr. Auerbach attempted to find the common thread linking 9 unrelated men, living in different cities across the United States, who had all died prior to 1982. The results of this study showed that the 9 men were connected through a single person they had met between 1979 and 1980 -- a handsome French-Canadian flight attendant with whom they all had sex. Gaetan Dugas was believed to have spread GRID across North America. Mr. Dugas, who the CDC designated as "patient zero", frankly admitted to having 250 partners a year. He was candid about his intentions of engaging in sexual activity with multiple partners in the cities that he would visit in the near future, and took no heed of the few purple KS lesions on his body. He believed that he had had more than 2500 partners since he became sexually active in 1972. By 1982, Dr. Auerbach could link over 40 cases of GRID from New York, Los Angeles, Miami, San Francisco, and Houston back to those 9 transmissions originating from Mr. Dugas.

In 1982, a new group, of heterosexual patients, began to report symptoms of GRID. These affected patients were intravenous drug users, of whom very few were homosexual men. In parallel, GRID symptoms emerged in a group of hemophilia patients from all over the United States. These patients had received repeated injections of Factor VIII, which was derived from the pooled plasma of thousands of donors. The medical community quickly realized that the predominant blood donors during the period had been intravenous drug users attempting to sell their blood. Soon after, the CDC released a report of GRID-like illness in a group of Haitians, among heterosexual non-intravenous drug using young males and females in Miami and New York. Suspicions that the virus may have originated in Haiti, and been brought back to the United States in the

1970s/early 1980s by homosexual men who frequently traveled to resorts in this region began to be articulated. Given the emergence of GRID-like symptoms in non gay populations, the CDC renamed the disease Acquired Immunodeficiency Syndrome (AIDS).

Given that AIDS was transmitted during homosexual and heterosexual contact, among intravenous drug users, and through the blood supply the causative agent had to be infectious. Dr. Luc Montaignier and associates at the Institut Pasteur identified the first potential candidate agent for this disease in 1983[2]. The group recovered a virus from the lymph node of a man who had the clinical symptoms of a syndrome they called Persistent Lymphadenopathy Syndrome (LAS). During the same period, Dr. Robert Gallo et al. also isolated a Human T-Cell Leukemia virus (HTLV)-like particle from an individual with AIDS in the United States[3]. In parallel, Dr. Jay Levy and co-workers identified a common retrovirus in asymptomatic and symptomatic AIDS patients and called the virus AIDS-associated retrovirus (ARV)[4]. Efforts were combined to identify if any relation existed between the viral particles isolated from these three sources. All three viruses had a clear tropism for CD4+ lymphocytes, proliferated well in these cells, and were not able to immortalize their targets[4]. The isolated viruses, contained a reverse transcriptase enzyme, and shared a number of other features with a previously described virus that caused severe immune debilitation, i.e. HTLV. Thus several groups, determined to find the causative agent of this AIDS disease, speculated that the causative agent of LAS was a member of the HTLV family of lentiviruses.

The scientific community was not satisfied with the idea that the causative agent of this disease was a member of the HTLV family. First, the AIDS causing virus had a

replication rate that was much greater than typical for this family of viruses[5]. Second, in contrast to the AIDS causing virus HTLV was seldom found in the plasma of infected patients[5]. Finally, the AIDS virus was cytopathic in CD4+ cells whereas HTLV, although also exhibiting CD4+ T cell tropism, was not[6].

Thus, through the concerted efforts of three research groups, it became evident that the viruses isolated by the separate teams were related and belonged to the same group of retroviruses, i.e. the lentiviridae family. Further investigation revealed that although the new virus cross-reacted with a number of HTLV proteins, it was clear that its proteins and the organization of the genome was different from that seen in members of the HTLV family. Given the discovery of a new virus, the International Committee on Taxonomy of viruses called the virus Human Immunodeficiency Virus (HIV)[7].

1.2 Origin of HIV

Although debates still exist on the subject, it is generally held that HIV and the simian immunodeficiency virus (SIV) may be linked to a single common ancestor that emerged around 1000 years ago[8]. Epidemiological and phylogenetic analyses suggest that SIV may have evolved into its human counterpart due to events occurring only 70 years ago[9]. In support of this hypothesis, both phylogenetic and demographic studies suggest that there is a high degree of probability that HIV-1 evolved due to the infection of humans living in western equatorial Africa by a closely related SIV strain that infects regional chimpanzees. The HIV-2 strains likely arose from the cross-species jump of SIV infecting Sooty Mangabeys living in western Africa into the human population in that region of the continent[10]. Moreover, the existence of the M, N and O groups, supports that SIV may have been transmitted to humans multiple times throughout

history allowing viral subspecies to diversify independently[10]. Efforts aimed at establishing the chronological moment of the cross-species jump based on evolutionary patterns in samples in comparison to the reference strain from a patient drawn in 1959, places the first transmission to humans around 1931 (1915-1941)[9]. Yet this model does not exclude the possibility that the jump from chimpanzees to humans may have occurred earlier.

Three theories have been proposed to explain the emergence of the HIV epidemic. The "Transmission Early Hypothesis" posits that the virus was transmitted in the 1800s or 1900s while humans were hunting chimpanzees for food. The infection remained isolated in small communities, until the 1930s. Then due to socioeconomic and political changes in Africa in the 1930s, it began to spread and diversify[11]. The second theory, the "Transmission Causes Epidemic Hypothesis" proposes that the virus was in fact transmitted from chimpanzees to humans in 1931, and began to spread and diversify in human populations from that date on. Finally, the "Parallel Late Transmission Hypothesis" suggests that multiple strains of SIV were transmitted during the 1940s and 1950s, which may have occurred via contamination of polio-virus vaccines with different SIV strains administered in Africa in 1957-1960[12]. Yet demographic data and phylogenetic analyses appear to be inconsistent with the spread of the infection via contaminated oral polio vaccines[13,14]. Three separate international laboratories did not find HIV in vaccine stocks and mitochondrial analyses on vaccine stock preparations revealed that vaccines were prepared on monkey tissue and not on chimpanzee cells. Both these findings have discredited "Parallel Late Transmission Hypothesis" as the possible explanation for the emergence of HIV by most experts.

Of the three hypotheses, the Transmission Early Hypothesis seems to be the most credible as it is supported by diversification, historical, sociological and demographic data. Based on sequence analysis, it is plausible that communities remained closed for several decades after the initial transmission of the virus from primates to humans. The virus, within closed communities, may have evolved and diversified into a small subset of clades[11]. As travel became an important feature of the African workforce in the late 1940s and early 1950s, men would leave their villages at the beginning of the week and return only at the end of the week[1]. Away from their families for extended periods of time, these men may have engaged in sexual activities with residents of the city, prostitutes, and travelers. Thus small rural villages potentially harboring this viral infection could have disseminated the disease through these cities, throughout Africa, and via travelers to the rest of the world. Thus an explosion of infections from the 1950s-1970s during the sexual revolution and globalization could explain the rapid spread of the epidemic across the globe.

1.3 Epidemiology:

Extensive worldwide spread of HIV started in the mid to late 1970s. In less than three decades, during the first of which it was still unknown, this virus caused the first modern pandemic. Epidemiological data compiled and disseminated by UNAIDS show that, at the end of 2002, 42 million people are infected worldwide with HIV (figure 1); 5 million were newly infected last year; and 3.1 million people died due to the disease. Moreover, the impact of the disease has left 13 million children orphaned, and has had a significant impact on the economic conditions of affected countries[15].

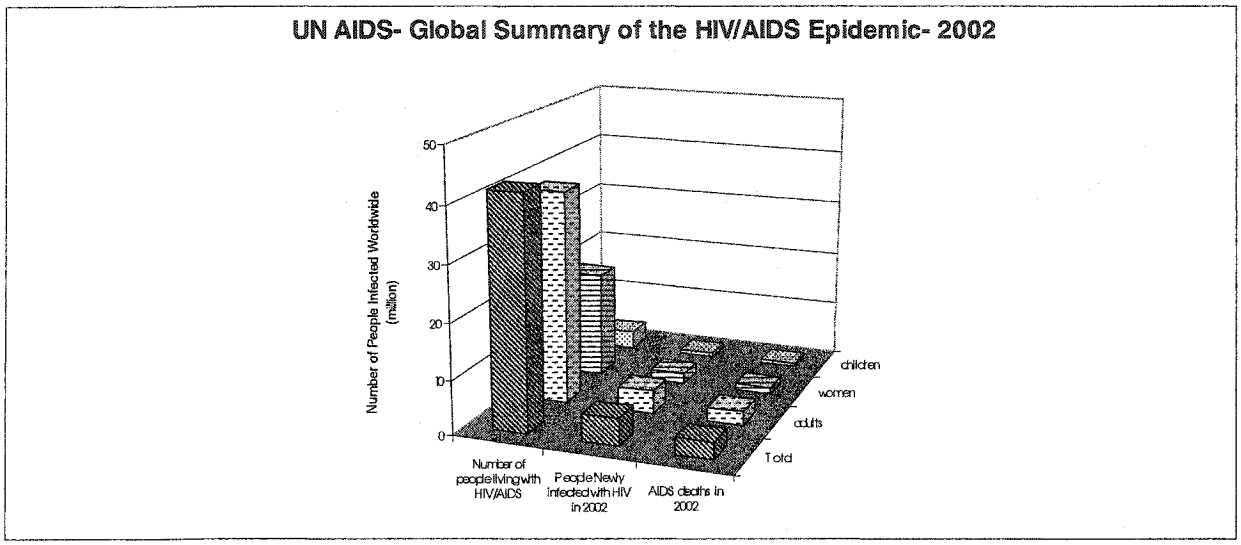

Figure 1. Summary of the HIV epidemic world-wide

Sub-Saharan Africa has been heavily affected by the pandemic with 8.8% of adults, 29.4 million people, infected with the virus(figure 2)[15].

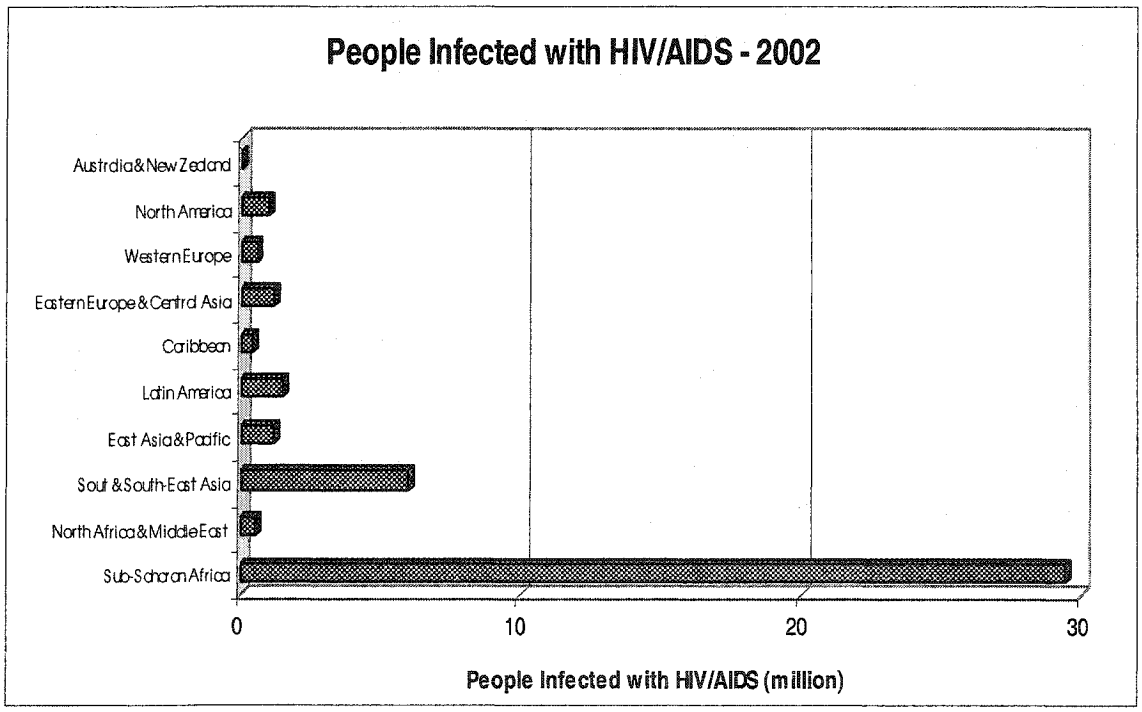

Figure 2. Epidemic by geographic area.

Yet despite efforts to curb the epidemic in this region of the world, the numbers still continue to rise due to heterosexual transmission in poor socio-economic conditions.

There is evidence for increasing HIV transmission in Eastern Europe, Asia and the Pacific. These regions are experiencing a rapid rise in the number of infections due to both heterosexual transmission and intravenous drug use. Public health data estimate that there will be an overwhelming number of new cases in all these regions in the coming years.

1.4 Virion Structure:

HIV virion structure is characteristic of the lentivirus family with a cone-shaped core (figure 3)[16]. The enclosed capsid area holds two identical strands of viral RNA in association with the viral RNA-dependent DNA polymerase (reverse transcriptase [RT]), the nucleocapsid proteins (p9 and p6), Tat, Vif, Nef, Vpr, viral integrase enzyme [IN], and viral protease [PR][16]. The core protein, p17 or matrix protein (MA), forms and provides structural support for the viral particle[17] and serves an essential role in nuclear localization of the pre-integration complex (PIC) and budding of viral particles[18]. Given the incorporation of all these proteins into the viral particle, it suggests that these proteins may all be necessary for early events in the viral life cycle[16].

The virion surface is usually composed of 72 trimers or tetramers of the envelope glycoprotein[19]. The envelope glycoprotein (Env, gp160) is cleaved in the Golgi into a complex, which consists of two portions, an external gp120 molecule and a transmembrane internal gp41 moiety[20]. The two glycoproteins are bound to one another through a small region of the transmembrane portion of the gp41, which noncovalently associates itself with the gp120 protein.

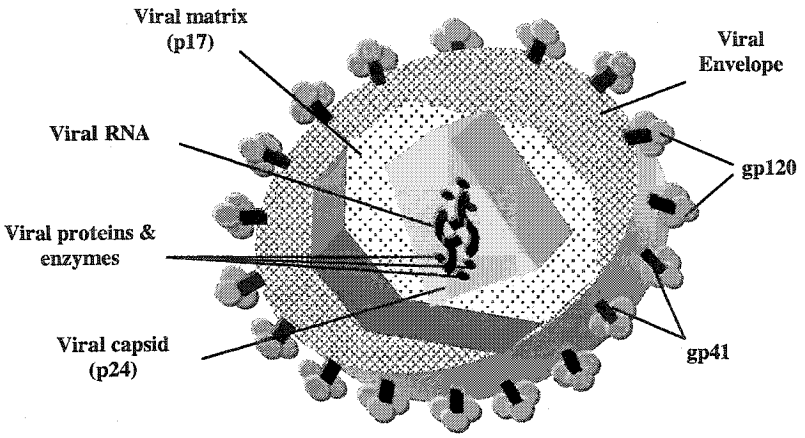

Figure 3. **HIV Structure**

1.5 Genomic Organization

The HIV genome is 9.8kb in length (figure 4). The earliest viral mRNA species are multiply spliced transcripts that give rise the viral regulatory proteins Tat, Rev, Nef, Vif and Vpr[16]. Rev interacts with a cis-acting RNA loop structure, which permits the production of gene products from full length transcripts that give rise to structural proteins. Unspliced, full length mRNA species give rise to the Gag precursor p55, which contains smaller p24 [capsid, CA], p17 [matrix, MA], p9 and p6 proteins. The Pol gene products are produced from the same mRNA species, via a frameshift. The precursor Pol protein is cleaved into viral RT [p66], protease [p10], and integrase [p32]. Gag and Gag-Pol transcripts are produced in a ratio of 20:1[21]. The Env gene product gp160, derived from an unspliced mRNA, is cleaved into the two envelope proteins.

Spliced mRNAs are responsible for the production of a number of key regulatory and accessory proteins. Tat is responsible for viral DNA transcription/elongation. This protein is critical for the recruitment of a number of cellular proteins such as cyclin T and cyclin-dependent kinase 9 (cdk-9) to the HIV long terminal repeat (LTR)[22] which are

integral for the effective assembly and activity of the reverse transcription complex (RTC). Nef (or negative factor) has proven to be a key viral particle in establishing a cellular environment that is conducive to viral replication. This protein is integral for the upregulation of several activation pathways[23], to the decreased expression of major histocompatibility complex (MHC) class I A and B[24], decreased expression of CD4[25], increased production of virions[26], and both the induction[27] and the prevention of apoptotic cascades[28]. Vif (viral infectivity factor) appears to be critical in circumventing the effects of cellular inhibitory factors allowing for effective reverse transcription within the hostile cellular environment[29]. Vpr (or viral protein R) plays an important role in nuclear localization of the PIC[30] and in the infection of resting macrophages[31]. Finally Vpu enhances the ubiquitination and subsequent degradation of CD4 molecules within the endoplasmic reticulum (ER)[32] and may also be important for virion budding[33].

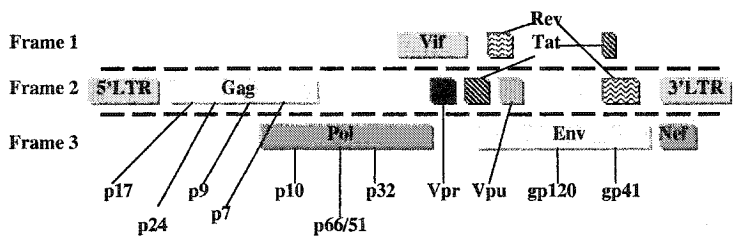

Figure 4. HIV genomic organization

1.6 Viral Life Cycle:

The viral life cycle begins with the interaction of the trimeric gp120 on the surface of an HIV virion with CD4 molecules on the surface of its target cell (figure 5-step 1 on page 24)[34,35]. This interaction results in a conformational change in the gp120, which reveals domains in gp41 necessary for interaction with HIV co-receptors located on the cell surface[35,36]. Although the gp120 complex can interact with up to a dozen

potential co-receptors, CCR5 (macrophage tropic virions) and CXCR4 (T cell tropic viral particles) are the most commonly used for this purpose[37]. The critical importance of these co-receptor molecules in the viral life cycle was demonstrated by the observation that persons homozygous for the deletion of a 32 base pair sequence in the CCR5 gene, that ablates the expression of this molecule, confers virtual resistance to HIV infection[38]. The interaction between Env and CCR5 or CXCR4 promotes the successful fusion of the virion membrane to the cellular membrane allowing the viral particle to enter the cellular cytoplasm[39].

Once the virus has entered the cell, the RTC consisting of the two viral RNA molecules, the initiating tRNA, RT, IN, MA, CA, Tat and Vpr are released from the cellular membrane (figure 5-step 2)[40]. This complex is subsequently stabilized by the Vif once the complex safely docks on the actin cellular skeleton[29,41]. Following the effective reverse transcription of the viral RNA, the PIC is assembled composed of the viral cDNA and all but the nucleocapsid protein found in the RTC, which subsequently targets entry into the nucleus(figure 5- step 3)[42,43]. MA[43], IN[44] and Vpr[45] possess nuclear localization signals ensuring the transport of the PIC into the nucleus. The viral genome may integrate into the host genetic material at a number of different sites, allowing for the integration of several proviral transcripts within the same cellular nucleus (figure 5- step 4)[46,47].

Tat protein, a member of the PIC, only plays its role once integration has taken place. Tat recruits cellular DNA polymerase and cofactors to the viral LTR leading to transactivation of the viral genome(figure 5- step 5)[22]. Earliest transcripts of the viral genome include the production of viral regulatory genes such as Rev, Tat and Nef[48]. At this early stage of replication, Nef mRNA predominates as 80% of the transcripts encode

this gene product[49]. Significant production of Tat enhances preferential transcription of the viral genome[50].

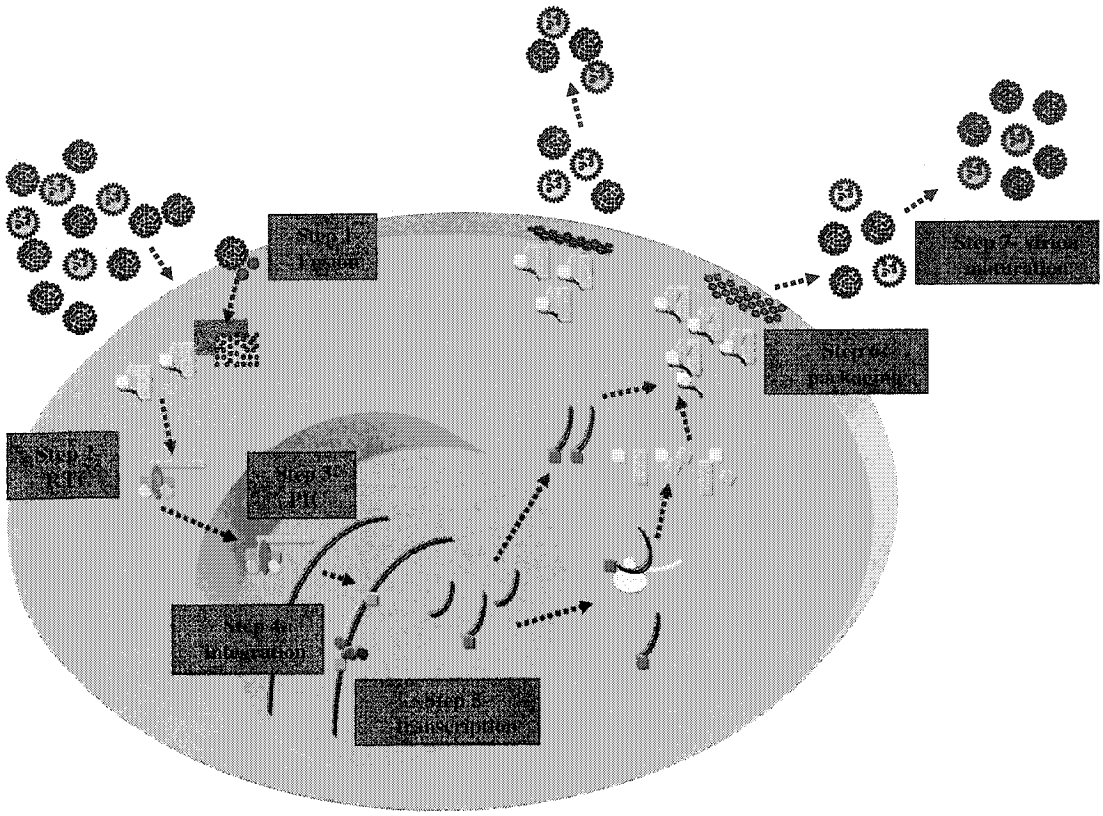

Figure 5. The HIV viral life cycle.

Increasing concentrations of the Rev protein, containing a nuclear export signal, allows for the export of unspliced mRNA species, harboring the sequences for viral structural and enzymatic proteins, from the nucleus to the cytoplasm for translation[51,52].

Assembly of viral particles occurs at the cell membrane (figure 5- step 6). Gag-Pol, Gag and Env proteins all have an important role in recruiting viral RNA, recruiting Vpr and ultimately in budding[53]. Viral release appears to preferentially occur in particular areas of the lipid bilayer that are rich in cholesterol[54]. Incorporation of PR in the virion is critical to complete the extracellular maturation of the virus particle (figure 5- step 7)[16].

1.7 The Immune Response at a Glance:

The immune system plays the integral role in protecting a host's internal environment from foreign invaders. The system involves a large network of cells and compartments which are all fine-tuned for their role in this task. Two lines of defense exist within this system, the innate immune response and the adaptive immune response. The first line of defense against foreign invaders includes cells involved in innate immunity[55]. Cells such as macrophages, natural killer (NK) cells, and granulocytes are capable of identifying highly conserved molecules located on the surface viruses, bacteria or parasites. Yet some pathogens may not alert this arm of the immune response, possibly due to the fact that they may not express these molecules on their surface or may have developed mechanisms to hide from this arm of the immune response. Thus the second line of defense, the adaptive immune response, may be particularly important in clearing these types of infections. B and T lymphocytes act as the key responders in this second line of defense, and tend to provide life-long immunity against the pathogen once cleared[55,56].

As lymphoid tissues filter and trap circulating antigens from interstitial fluid surrounding diverse tissues and blood, these sites act as a milieu for the convergence of foreign antigens and circulating lymphocytes[55]. Regardless of the site of infection, foreign antigen presented on non-lymphoid cells within these sites can induce the maturation and the induction of the adaptive immune response within the peripheral lymphoid organs. Given the expression of a number of specialized adhesion molecules on the surface of resting lymphocytes that allow for transcytosis across endothelial cells, resting lymphocytes continuously traffic between the blood and lymph[55,56]. Once a

lymphocyte recognizes its cognate antigen in the lymph node, it is trapped until it proliferates and differentiates into a mature cell capable of combating the infection[57].

In an attempt to cover the wide spectrum of potential infiltrating foreign antigens, the immune system produces lymphocytes each bearing a single specificity. The generation of these unique specificities occurs via complex genetic events in the bone marrow (B cells) and thymus (T cells) that lead to the creation of an enormous number clones with unique antigen binding characteristics. Naïve lymphocytes are subjected to rigorous selection processes in their compartment of origin to ensure that cells recognizing self are not released into the periphery[55,56]. Most of these cells are deleted such that cells recognizing foreign antigens enter the circulation where they can encounter their cognate antigen, proliferate and differentiate into effector cells.

Antibodies, produced by B cells, can function in the effector phase of immune responses by 1) neutralizing pathogens and in some cases toxins secreted by pathogens, 2) serving as opsonins that promote phagocytosis of foreign material by macrophages or neutrophils and their eventual degradation within these cells, 3) initiating the complement cascade thereby activating several mediators that promote inflammation and assembly of the membrane attack complex, 4) serving as a bridge between a pathogen and NK cells in antibody dependent cellular cytotoxicity. Only pathogens located in the blood or the extra cellular spaces are accessible to antibodies[55]. Some bacteria and all viruses replicate inside cells where they cannot be detected by this component of the adaptive immune response. T lymphocytes, as the effectors of the cell mediated immune response, are responsible for the destruction of intracellular pathogens[55,56]. Cell mediated immunity relies on the direct interaction between infected cells and the T cells[57]. Recognition of

infected cells by T lymphocytes initiates a series of events that result in the destruction of infected cells. T cells also contribute to the destruction of extra cellular pathogens by secreting factors that activate B cells and macrophages[56]. The activation of T cells depends on the presentation of small peptide fragments of foreign antigen in the context of specialized cell-surface molecules, i.e. the membrane bound glycoproteins encoded by genes within the major histocompatibility complex (MHC) expressed on the infected host cells[55].

1.7.1 The Major Histocompatibility Complex:

Two classes of MHC molecules, class I and II, are important in this process (figure 6)[55]. MHC class I antigens can be found on the surface of all nucleated cells and are recognized by CD8+ T lymphocytes (cytotoxic T lymphocytes-CTL). MHC class II molecules are expressed on the surface of professional antigen presenting cells (APC) and are recognized by CD4+ T lymphocytes (helper T cell-Th)[55-57]. The dichotomy of the system serves to target peptides derived from pathogens or foreign material localized in different cellular compartments, via MHC class I or II molecules. In general, peptides from exogenous antigens are presented by MHC class II alleles to Th cells, and peptides from endogenous antigens are presented on MHC class I molecules to CTLs. This notion of the division of antigens presented by MHC class I and II to the immune system is not absolute, as presentation of peptides from particular compartments can occur through either pathway, indicating the potential for cross-talk between the two routes of antigen presentation[58,59].

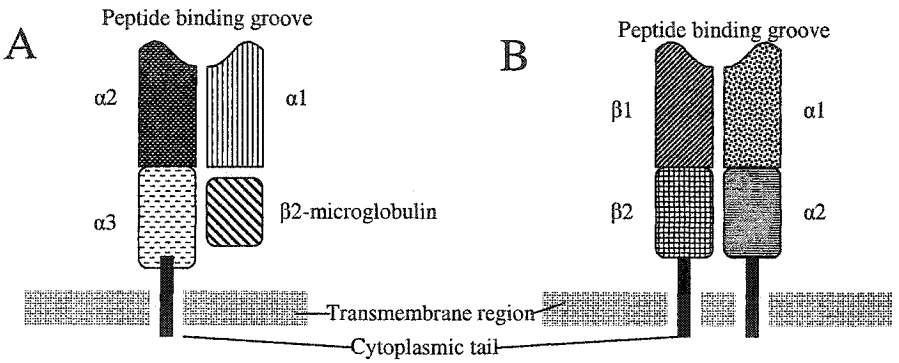

Figure 6. Major Histocompatibility Complex (MHC) class I (A) and class II (B) structures.

MHC is highly specialized to deal with a wide spectrum of foreign invaders due to its polygenic (multiple molecules expressed within the same individual) and polymorphic (multiple genes within the population) nature. The MHC locus contains more than 100 genes located in human chromosome 6[60]. The α chain for the MHC I and the α and β chains for the MHC II molecule are encoded in this region, while the β_2-microglobulin chain that associates with the class I molecule is encoded on a separate chromosome (figure 6A)[55,60]. The human class I region contains three loci, called HLA-A, HLA-B and HLA-C, each encoding the heavy chain of a classical MHC class I antigen. Non classical MHC class I alleles are also encoded by other loci within the MHC and in some cases have a more limited tissue expression pattern than do classical MHC class I antigens[56]. The HLA-D region of the human MHC encodes at least six α and ten β chain genes for MHC class II molecules. Three loci DR, DP, and DQ encode the predominant class II alleles, although other genes encoding such molecules have also been identified[56]. The polygenic nature of this locus allows for the presentation of peptides able to form complexes with the products of any one of the alleles encoded by expressed MHC class I and II genes. Moreover, these loci are highly polymorphic, and

the great diversity in these loci, with more than 80 alleles for the HLA-A, 180 for the HLA-B and 40 for HLA-C, (and at least equally high levels of polymorphism for HLA-DR, DP and DQ), leads to diverse patterns of expression of these alleles in different populations[55,56,61].

Distribution of the MHC molecules on the surface of cells is directly related to a cell's function and its role in infection. MHC class I molecules are expressed on all nucleated cells, as mentioned earlier. Thus any nucleated cell that becomes virally infected or transformed to express altered self antigens can present peptides to circulating T cells[55,57]. Professional APC (B cells, macrophages and dendritic cells) also express MHC class II molecules. These can present foreign peptide antigens to CD4+ T cells, which secrete cytokines upon activation[55,57]. Cell surface expression of MHC molecules is influenced by cytokines in the cellular microenvironment. Of interest, interferon-γ (IFN-γ) is particularly effective in inducing the cell surface expression of MHC class II. This effector molecule has dramatic effects at several levels of antigen presentation, as it upregulates the expression of MHC class I as well as several other components that are crucial to antigen processing and presentation[55].

1.7.2 B-lymphocytes:

The B lymphocytes, which develop in the bone marrow, express a unique receptor on their surface (figure 7). A secretable version of this receptor having the same antigen binding characteristics is produced in large quantities by plasma cells that differentiate from B lymphocytes upon activation. This molecule, the antibody, or immunoglobin (Ig) is composed of two chains each containing two regions. The constant region (Fc) contains sequence information that determines the class of the antibody molecule. There

are 5 Ig classes, IgG, IgM, IgA, IgD and IgE that are also distinguished from each other by differences in effector function. The second portion is the unique component of the molecule, responsible for binding the antigen (Fab).

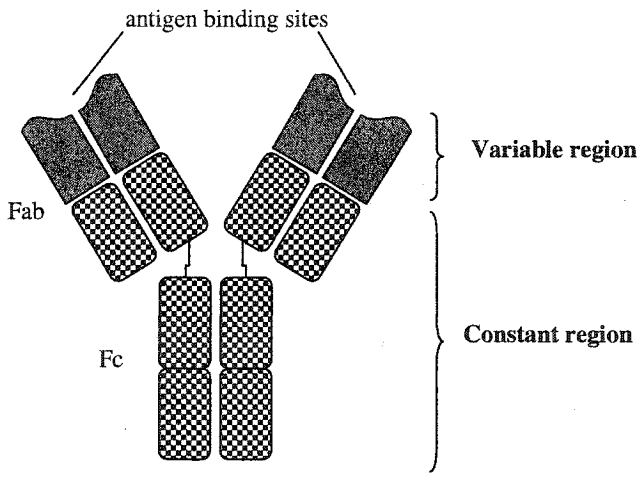

Fig 7. Antibody structure.

A number of irreversible events occur during B lymphocyte maturation that give rise to the great diversity seen in antibody specificities despite the limited number of variable region genes[55]. There are 5 events during B lymphocyte receptor rearrangement that are critical in conferring the unique properties of the antigen binding domain of each circulating lymphocyte. The first step in antibody production begins with the random recombination and rearrangement of segments within the inherited variable gene locus (figure 8A). The process requires the association of different V and J regions in the light chain, and V, D and J regions within the heavy chain. More than 100 V segments, 30 D segments, and 6 J segments are rearranged to give rise to unique combinations of the functional antigen binding regions in the heavy chain[56]. Similarly, multiple segments are equally recombined in the light chain to generate this level of diversity in the antigen binding cleft (figure 8A). The second level of variation is attributable to the random

endonuclease activity of the recombination activating genes 1 and 2 enzymes (RAG1 and 2) activities[55]. These enzymes splice DNA segments randomly leading to the inclusion or exclusion of short stretches of nucleotides that can impact heavily on the amino acid sequence and thus on protein structure (figure 8B). Thirdly, endonuclease activity may lead to the generation of gaps in the recombined sequences, leading to the incorporation of n-nucleotides (non-template encoded nucleotides) to fill the gaps.

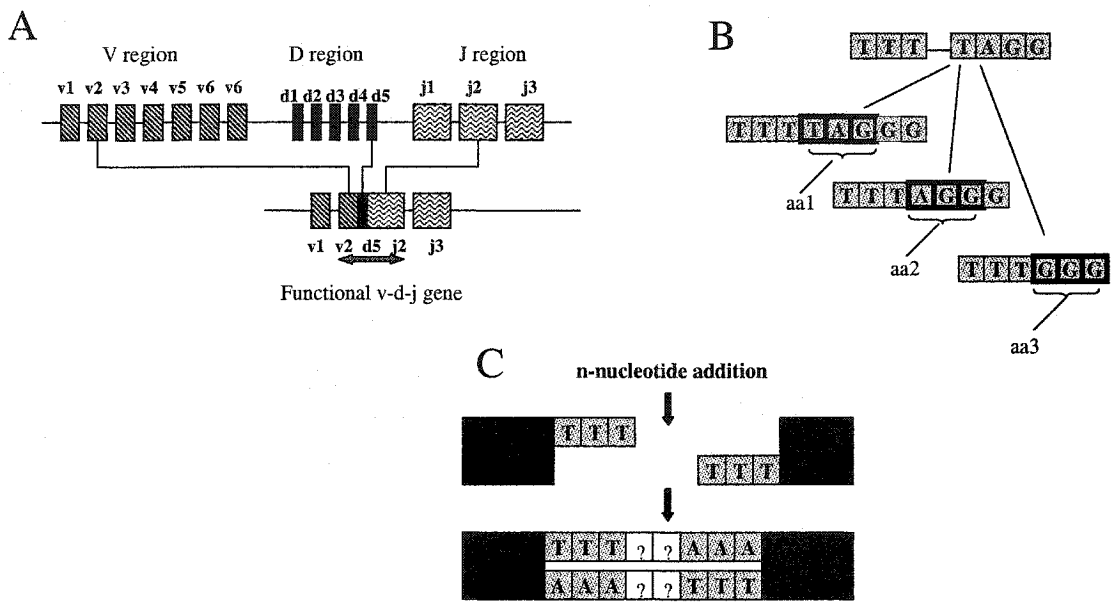

Figure 8. Mechanisms involved in the generation of antibody diversity; random recombination of segments within the heavy chain (A), random endonuclease cutting (B), and the addition of n-nucleotides (C).

The terminal deoxynucleotidyl transferase enzyme (Tdt) adds up to 20 random nucleotides to the single-stranded ends of coding DNA after hairpin cleavage (Figure 8C)[55,56]. The random association of a heavy chain with either of the two light chains, the κ or λ light chain, leads to added diversity during lymphocyte development[57]. Finally, on antigen encounter in the periphery an additional mechanism, somatic hypermutation or affinitiy maturation, introduces a considerable number of point mutations within the variable region of the rearranged heavy and light chain genes. This process gives rise to

mutant antibodies that may bind antigen with a higher affinity than the previously rearranged antibody[55].

1.7.3 T-lymphocytes:

Unlike their B lymphocyte counterparts, T lymphocytes, developing in the thymus, do not secrete their receptor and cannot bind free antigen. Using their T cell receptor (TCR), T cells bind their antigen in the context of an MHC molecule on an antigen presenting cell (APC). T lymphocytes can be divided into two classes based on the expression of CD4 or CD8 cell surface molecules[55,57]. CD4 molecules enhance the association of TCRs and MHC class II molecules, while the CD8 molecule encourages the interaction of TCRs with MHC class I molecules. Thus CD4 and CD8 are integral for the promotion of the contact between different subsets of T cells expressing TCRs and the MHC expressing cells[55].

Similar to the B cell, each T cell has a unique TCR. This receptor is composed of two separate chains, an α and a β polypeptide chain (figure 9). Diversity in the TCR is generated via the recombination of a limited number of segments that generate the variable region of the TCR, as mentioned above for antibody generation minus peripheral affinity maturation. Thus rearrangement and recombination of different segments of V and J segments in the α chain, and V, D, and J segments in the β chain, random endonuclease activity, addition of n-nucleotides, as well as the random association of different α and β chains leads to the production of a vast number of lymphocytes each bearing a unique TCR molecule[55,56]. Once successful β gene rearrangement has occurred, the β polypeptide is presented on the cell surface in conjunction with a generic pre-T α chain. Successful signaling via this complex allows the cells to proliferate. These T cells,

all expressing the same β chains, then begin to undergo somatic rearrangement at the α chain locus. This process leads to the generation of T cells expressing different α chains in conjunction with the same β chain[56,56].

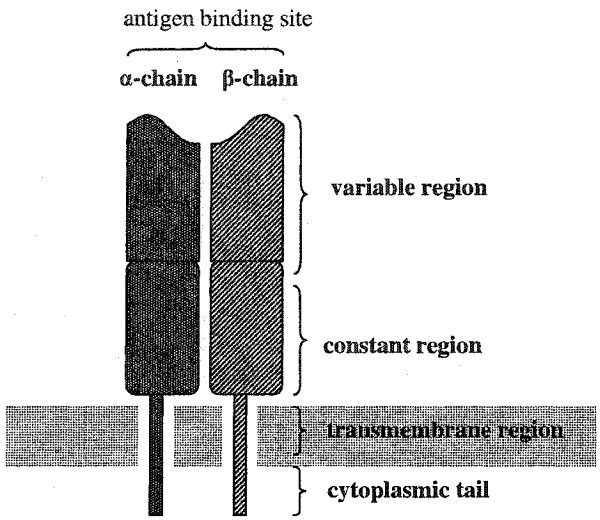

Figure 9. T cell receptor (TCR) structure.

The function of the TCR relies on associated transmembrane proteins (δ, γ, ε, ζ chain) which all together form the CD3 surface complex[55]. Together, the complex is capable of transducing a signal when the TCR recognizes its cognate antigen in the context of the MHC. This interaction subsequently leads to activation, maturation, proliferation, and the secretion of various effector molecules.

1.7.4 Lymphocyte trafficking:

Naïve lymphocytes trafficking through lymphoid organs may encounter professional APCs displaying their cognate antigens. Recognition leads to the retention of the lymphocyte in the organ, allowing the stimulated cell to enlarge and begin dividing[55]. Given that a tremendous number of unique lymphocytes exist in the circulation at any given time, it is clear that those that encounter their specific antigen must proliferate and

mature in order to generate sufficient effector cells to target the foreign agent. Thus clonal expansion allows for the specific proliferation of up to 1000 daughter cells from a single naïve lymphocyte recognizing its cognate antigen[55]. Proliferation continues for 4-5 days, at which point the daughter cells become mature effector cells. Most effector cells are subject to a pre-determined life-span, and thus begin to die via apoptosis[55,56]. A small subset of daughter cells persist, allowing for the development of immunological memory to the specific activating antigen. If antigen is reencountered memory cells will respond with a shorter lag phase than occurred following first encounter with the antigen[55,61]. This leads to a more rapid induction of the effector phase in the secondary than in the primary immune response. In B cells this results in rapid production of higher levels of antibody with a higher affinity. For T cells this also results in more rapid, potent and focused effector responses[55].

1.7.5 Antigen Processing and Presentation:

Identification of some of the key players in antigen presentation and processing came as a surprise. It was assumed that the antigen processing machinery had to be present in all cell types that co-expressed MHC class I antigens. The machinery also had to have access to peptides from a wide range of cellular compartments. Based on the knowledge that all cellular proteins are subject to continuous turnover mediated by the cellular proteasome found in all cell types, it was hypothesized that this protein complex may also play a role in antigen processing[58,59]. Experiments using inhibitors to block proteasome activity also prevented MHC class I maturation and antigen presentation on the cell surface[59]. Thus it became clear that that the machinery involved in the normal cellular proteolytic pathway was also responsible for the production of peptides to be

displayed to the immune system. Evidence for the role of the proteasome in the immune response was reinforced by the discovery of a number of IFN-γ inducible proteasomal subunits that are transcribed and translated during an immune response, and are essential in the activity of the "immunoproteasome"[58].

Although the proteasome is integral in the generation of cytosolic peptides for presentation to cells of the immune system, other key cellular players are involved in this process. For example mechanisms involved in proteosomal targeting of proteins, translocation of peptides from the cytosol, peptide loading, and MHC class I maturation all appear to play an important role in shaping the subsequent immune response.

1.7.5.1 Ubiquitin Complex:

Antigen presentation begins with a complex network of proteins involved in targeting proteins for degradation, the ubiquitin protein ligases (figure 10)[62]. Initially, ubiquitin is activated by the E1 enzyme that forms a thiol ester on the targeted protein's C-terminus and then transfers the protein to a ubiquitin-carrier protein (E2)[63]. Activated ubiquitin is then ligated by a ubiquitin-protein ligase (E3) to the epsilon group of lysine via an isopeptide bond on a protein substrate or to a ubiquitin moeity already bound to the protein[63]. Thus a polyubiquitin chain is sythesized, which allows the 26S proteasomal complex to recognize the protein as being ready for degradation[59]. As the proteasome degrades proteins non-specifically, it is probably the ubiquitin system that confers a level of specificity that marks proteins for degradation. Cells contain one E1, 10-15 E2s, and over 15 E3s[64]. Combinations of different E2s and E3s can preferentially ubiquinate certain proteins. The specificity of this system has been demonstrated in targeted

degradation of cellular machinery during cell cycle and signal transduction, such as the degradation of cyclin-dependent kinases[65] and the inhibitor of NFκB, IκB[66].

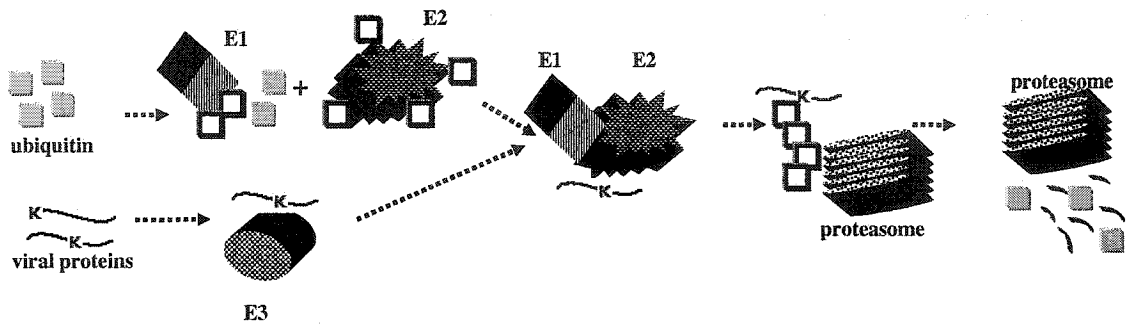

Figure 10. Ubiquitin dependent protein degradation pathway.

Ubiquitination by the E2/E3 complex relies on the composition of the N-terminal residues of the target proteins, i.e. the "N-end Rule"[67]. The N-end rule dictates that large bulky or charged amino termini are ubiquitinated rapidly and thus targeted for fast degradation. Foreign antigens, that are ubiquitinated more quickly, appear to be presented more effectively, and to elicit stronger immune responses[67,68]. In addition when the E3 ubiquitin ligase function is inhibited, no antigen presentation occurs supporting an integral role for the ubiquitin system in antigen processing and presentation[69].

1.7.5.2 The Proteasome:

The next step in the process involves recognition of the ubiquitinated protein by the proteasome (figure 10). The proteasome is a large complex of proteins that is responsible for protein turnover in the cell and is found in the nucleus and the cytoplasm of all eukaryotic cells[70]. The 20S core, ~700 kD in size, appears as a cylindrical structure composed of four stacked rings. Seven homologous, but distinct β subunits, surround the central chamber, of which 3 units in each ring are responsible for the proteolytic activity.

The outer rings are composed of 7 homologous but distinct α-subunits, which guard the substrate entry site of the proteasome[70].

The proteasome is considered to be a "multicatalytic protease" as it exhibits 3 peptidase activities: "chymotrypsin-like" activity cleaves after large hydrophobic residues, "trypsin-like" activity, cleaves after basic residues, and finally an acidic activity that cleaves after acidic residues. The specific activity depends on the β subunit, which is responsible for the peptidase activity[71].

At each end of the proteasome complex there is a 19S subunit responsible for recognition of ubiquitinated substrates. This regulatory complex, contains the sites for binding of the polyubiquitin chains, and degrades these chains as the protein is processed, releasing free ubiquitin into the cell[70]. Additionally, the 19S regulatory subunit is required for protein unfolding, and is thus a critical component of the 26S complex[72]. Proteins are digested in a highly processive manner once they have entered the complex. Seventy percent of the proteosomal degradation products are smaller than 8 amino acids in length, 20% are longer and yet could still be trimmed by additional proteases to fit in the MHC class I groove, and only 10% of the products of the proteasome are 8-mer peptides[73]. Proteasome cleavage is responsible for the synthesis of the C-terminus of the antigen[74]. This is clear as few proteases can remove the C-terminal residues from antigenic peptides.

Two of the proteasome β subunits are encoded within the MHC locus. These subunits enhance the presentation of some antigens[75]. The 2 β subunits, LMP2 and LMP7, are constitutively expressed in lymphoid tissues, and are strongly induced by IFN-γ in non-lymphoid cells[76]. Proteasomes containing LMP 2 and 7 are called

"immunoproteasomes" as they have an important role in antigen presentation. LMP2 and 7 induction by IFN-γ results in the incorporation of these subunits into cellular proteasomes, thus leads to the replacement of the constitutively expressed homologues. Induction of these two subunits leads to an increase in the trypsin-like and chymotrypsin-like activity of the proteasome[77]. With the augmentation of these two activities a greater proportion of peptides are generated with basic or hydrophobic C-termini, which are required for efficient transport by the transporters associated with antigen processing (TAP) proteins, and binding to MHC class I molecules[78]. The newly formed immunoproteasome seems to be selective and is more effective in the presentation of certain immunogenic peptides[75].

1.7.5.3 TAP Transporter:

Studies aimed at delineating the antigen presentation pathway in the mid 1980's used two mutant cell lines with low levels of MHC class I on their surface, RMA-S and T2[79]. Unstable MHC class I complexes accumulated in the ER in both these cell lines, which led investigators to infer that the defect in MHC class I expression was in peptide translocation from the cytosol to the ER. The genetic defect was characterized, and mapped to a region of the MHC class II locus that encoded for two proteins, TAP I and II[80]. Introduction of both TAP I and II into the cell lines by transfection, restored MHC class I expression on the cell surface. It was therefore conclusively accepted that these two proteins serve an integral role in antigen presentation.

Based on certain calculations, Yewdell et al. concluded that many more proteins are degraded than are presented by the immune system[81]. It was apparent that as few as 1 in 1000 peptides synthesized in the cytosol by the proteasome were actually presented by

an MHC class I complex[82]. Restriction of peptide presentation can occur at the level of TAP translocation activity[83], inefficient MHC class I loading[77] and exclusion of peptides with a proline at position 2 from the ER by the TAP system[84].

Peptide binding to the TAP protein-binding site is the first step in the process of antigen translocation. TAP translocates peptides of 8-16 amino acids in length most efficiently[85]. The C-terminal residue of the peptide being translocated appears to influence TAP translocation. Hydrophobic C-termini[86] and proline at position 3 in the peptide[87] increase translocation efficiency, whereas acetylation or methylation of the N-terminus or conversion of the C terminal carboxyl group of a peptide to an amide decrease TAP binding and thus translocation to the ER[88].

As only 10% of peptides degraded by the proteasome are 8-10 amino acids long and 20% are longer than the optimal length required for binding to the MHC class 1 binding groove, the majority of peptides that are translocated by the TAP transporters require further trimming. A variety of amino-peptidases exist in the ER, that allow for the modification of the length of the peptide[59]. The complex expression of different combinations of proteases, depending on tissue expression, may determine the degree or type of antigens presented to the immune system. Thus it is possible that immunodominant epitopes may be translocated as longer pieces, and then trimmed to the optimal size in the ER[58].

1.7.5.4 MHC Class I peptide loading:

Polymorphisms in HLA molecules lead to the generation of molecules with different binding grooves for peptide presentation. Six pockets have been identified within the MHC class I molecule binding grooves, unique to each HLA allele[55]. Binding

grooves vary both in depth and chemical interactions with peptides, and therefore selectively present those peptides that conform to the requisite shape of the groove and that bind tightly. Peptides form salt bridges and hydrogen bonds within the two pockets located at either end of the groove. Binding within these pockets is crucial for stability and maturation of the MHC class I-peptide complex and cell surface expression. Given structural similarity in the binding grooves of certain HLA molecules, it is possible to group some HLA alleles into supertypes such as the HLA-A3 supertype, which includes HLA-A3, A11, A31, and A33. Members of a supertype all bind similar peptides with common HLA binding motif sequences[89,90,91]. Binding of peptide is essential for the maturation of MHC class I complexes[92]. Once the MHC class I heavy chain, β2-microglobulin, and peptide complex are assembled, the complex is released from the TAP transporters in the ER, and shuttled, via the translocon, through the Golgi apparatus to the cell surface[93].

1.7.5.5 The Alternative Pathway of MHC Class I loading:

While the MHC class I pathway presents peptides from the endogenous pool of cellular proteins, the MHC class II pathway presents peptides derived from exogenous antigens to the immune system. MHC class II is expressed on professional APCs such as macrophages, monocytes, dendritic cells, and B cells[55]. Exogenous peptides, picked up in endocytic vesicles, are cleaved in acidic compartments by cellular proteases, and are then loaded onto the MHC class II molecules in specialized MHC class II compartments (MIIC) [58]. The MHC class II molecule is associated with an invariant chain (Ii) that blocks the binding of peptides found in the ER. Once an appropriate peptide from an

endocytic vesicle arrives in the MIIC compartment, the Ii is dislocated, the peptide binds, and the complex is escorted to the cell surface by a chaperone protein, HLA-DM[55].

Exogenous proteins can also be presented by the MHC class I pathway. Two potential routes include: 1) exogenous peptides having access to MHC class I loading sites in the ER, or 2) a post-Golgi loading of exogenous peptides that compete for the binding site of the endogenously loaded peptides from the ER. A number of possible mechanisms for this cross-talk between the two pathways have been identified. Isenman et al. demonstrated that antigens from endocytic vesicles can be "transferred" to the cytosol, in a manner that does not disrupt the endosomal membrane[94]. These proteins can then be degraded by the proteasomal pathway, and presented through the MHC class I pathway as endogenous proteins. A second route of cross- talk was proposed where endocytic vesicles that are overloaded and overwhelmed by their contents can rupture within the cell, allowing exogenous antigens to be released into the cytosol[95]. Finally, MHC class I molecules that are internalized from the surface into endosomal compartments already loaded with a peptide from the endogenous pathway, under low pH, may loose their peptide from their binding groove. Thus exogenous peptides found in these endosomal compartments may associate with these empty binding grooves, and allow for the re-externalization of the MHC I molecules complexed to peptides derived from exogenous antigens[96]. Sub-optimally loaded MHC class I molecules, have an additional chance to exchange their peptides for exogenous peptides that bind with higher affinity to the peptide that was loaded in the ER upon arrival in the Golgi[58]. Antigens processed in endosomal compartments are degraded by a separate class of proteases, and thus allow for a diversification of potential peptides. The ability to present exogenous

peptides on MHC class I molecules, may be a feature of professional APC[58]. It is additionally possible that the nature of the antigen acquired by an APC from its microenvironment may influence the path by which it is presented[97].

1.7.5.6 Mechanisms used by viruses to evade processing and presentation

As a number of viral infections take place in the cytoplasm, viral proteins, produced in the cytosol are inevitably targeted to the MHC class I pathway. Families of viruses have developed sophisticated methods of avoiding detection by the immune system, by altering the ability of the MHC class I pathway to warn the immune system of their presence within a cell. Oncogenic adenoviral strain 12 artfully represses the transcription of a β subunit of the immunoproteasome, TAP proteins and of the MHC class I heavy chains[98]. Similarly, in an effort to go undetected, Epstein-Barr virus (EBV) contains a glycine-alanine repeat within its gene products that inhibit antigen processing[99]. Cytomegalovirus (CMV) equally evades the immune response via the rapid phosphorylation of its immediate early protein. This modification prevents the degradation of a critical immunodominant epitope located within the phosphorylated region[100]. Viruses such as the Moloney leukemia virus contain a single amino acid within a dominant epitope that prevents the epitope from being processed and presented by the MHC class I pathway[101]. Association of HIV-1 Nef protein with the cytoplasmic tail of MHC class 1 loaded molecules induces rapid internalization of these complexes making them unavailable for recognition be cognate T cells[102].

1.8 HLA and HIV

Several studies have focused on distributions of MHC encoded alleles and their role in HIV disease progression or protection from infection[103-108]. Large cohort studies have allowed for the evaluation of the role of HLA alleles in different groups of HIV infected patients, such as fast progressors or long term non-progressors (LTNP)[109]. As the disease is still relatively new, viral infection would have had little impact on the selection of HLA allele expression within the population. An interesting observation made in chimpanzees makes the role of HLA in the rate of disease progression difficult to explain. Chimpanzees, typically exhibit a benign course of disease, yet they share 98% homology with humans in their HLA allele sequences[110]. Given this degree of similarity between the two species, it is difficult to reconcile how these alleles, which are responsible for eliciting immune responses in both species, could have an impact in one and not the other.

Heterozygosity at MHC loci would increase the number of potential peptides presented to the immune system and therefore enlarge the breadth of the response. In fact, homozygosity was associated with a negative impact on disease progression[105]. The presence of certain alleles is associated with a more rapid disease course, while still others with progression. Several reports have shown that HLA-B35 and Cw4 are strongly associated with a poor clinical outcome, while B57, and B27 are associated with slow disease progression[105,111]. In several cases haplotypes that include several linked loci influence the strength of the association of particular MHC class I alleles with disease progression[111,112]. Although certain protective alleles such as B27, B57, B39, etc...are enriched in groups of LTNPs[106], the majority of these alleles appear to be rare in most

human populations. Thus populations of resistant individuals may emerge with time, due to evolutionary constraints placed upon survival by this new human viral infection.

A separate population of individuals, that are exposed-uninfected (EU), remain seronegative despite repeated exposures to the virus. Makedonas et al. have shown that EUs demonstrating HIV-specific effector responses appear to be at a 40 fold lower risk of seroconversion than EUs without antiviral activity[113]. Given that effector responses may be important for preventing infection, the role of HLA in this group has been the subject of extensive discussion. While the relative risk of expressing A2, A28, and DR13 in EUs may be elevated compared to infected individuals according to one study[112], the association of these molecules with protection is not clear in another[107]. Two groups of prostitutes in Africa[114,115] and in a group of discordant couples[116] appear to be protected from infection. It is possible that these individuals may be protected due to superior antiviral cytotoxic responses allowing them to neutralize the virus at exposure. Yet, an alternate explanation for this resistance may be HLA independent. The virion coat, as it buds off, incorporates host cell surface molecules such as HLA molecules[117]. It is possible that on infection, exposure to heterologous HLA molecules (derived from the infecting host) on the viral coat may induce an anti-HLA response in the newly infected subject. Perhaps, constant exposure to foreign cells, expressing heterologous HLA molecules due to continuous sex-trade or sexual contact with the same partner, may lead to effective allo-HLA responses in these three groups. Consequently, these responses may protect against infection as cytolytic activity or antibody mediated effector responses within the mucosa may lead to the clearance of non-host HLA carrying viral material[118].

1.9 The Immune Response to HIV:

1.9.1 NK Cells

NK cells, which serve as a first line non-MHC dependent defense against invading pathogens, are vital to the immune responses to viral infections and tumors[119]. These cells secrete antiviral cytokines such as IFN-γ, granulocyte/macrophage-colony-stimulating factor (GMCSF), tumor necrosis factor-α (TNF- α), RANTES, and MIP-1 α without prior stimulation[120]. NK cell activity is triggered via the recognition of reduced expression of MHC class I on the surface of infected cells[121]. Several viral infections have devised mechanisms to escape CTL detection via downregulation of MHC class I surface expression[122]. As mentioned previously, the HIV protein Nef is responsible for the retention of MHC class I A and B molecules within the ER, thus leading to reduced presentation of viral proteins to circulating lymphocytes[24]. Yet, in HIV infection HLA-C and E molecules are expressed at normal levels[123]. These molecules are the primary ligands of NK cell receptors. Depending on whether HIV infected cells expressing HLA-C or HLA-E encounter NK cells with stimulatory or inhibitory versions of these NK receptors, they will either be killed or ignored by NK cells[123]

Recent work has demonstrated that a strong association exists between the co-expression of KIR3DS1 and Bw4 and slow disease progression, supporting the notion that NK cells play an integral role in HIV disease progression[124]. NK cell function appears to be seriously impaired with progressive infection, despite preserved cell numbers[125]. Of note, a negative correlation appears to exist between CD4 T cell numbers and NK activity[126]. It is speculated that reduced NK activity may be attributable to a decline in IL-12 secretion with advancing disease[127]. Additionally, mature cytotoxic NK

cells expressing CD16/CD56 appear to fall in HIV infection, while weakly cytotoxic CD16-CD56- cells increase[128]. Moreover, a striking positive correlation exists between NK activity and viral load, indicating that greater viremia may lead to increased activation of this cell compartment[126]. It is believed that impaired NK cell function may impact heavily on CTL activity, as gradual NK defects may lead to a reduced production of IFN-γ, which is important for CD8+ T cell activity.

1.9.2 Macrophages

Macrophages are important players in the innate immune response to infection. Foreign material is actively engulfed by this subset of cells and digested independent of T cell help[55]. Yet several types of pathogens have developed mechanisms to avoid this activity, and thus specific immune responses to these infections are required for clearance. Macrophages present fragments of digested pathogen products complexed to MHC class I and II molecules to circulating lymphocytes. While some studies have alluded to reduced phagocytic activity in HIV-infected macrophages[129], this issue remains unclear[130]. Several groups have demonstrated that *in vitro* HIV infected macrophages are less effective at inducing lymphoproliferative responses[131], secreting cytokines[132], and responding to chemoattractive signals[133]. Recent work has also demonstrated the reduced expression of essential costimulatory molecules on APCs, CD40 and CD80/CD86, on the surface of these APCs[134,135]. It is still unclear if these defects are directly due to infection of the macrophages by HIV or due to aberrant signals received from other immune cells.

1.9.3 Dendritic Cells

Dendritic Cells (DC) are professional APCs that are critical initiators of the adaptive immune response[55]. Several different classes of DCs have now been identified. Each subset of DCs appears to be responsible for surveillance within distinct compartments. For example, while Langerhans cells are essential for immune-surveillance in the skin, myeloid DCs perform the same duty in the circulatory system[136]. Several subsets of DCs are known to express CD4 and both co-receptors (CCR5 and CXCR4) required for viral entry. Difficulty in identifying if DCs could be infected by the virus stemmed from the fact that the virus replicates at a very low level in this cell type. It is now certain that Langerhans cells, myeloid DCs, as well as plasmacytoid DCs can all be infected both *in vivo* and *in vitro*[137,138].

Discovery of the novel molecule, DC-SIGN, has confirmed the critical role of DCs in early events of HIV dissemination. This molecule appears to bind viral particles with a higher affinity than HIV co-receptors[139]. Additionally, DC-SIGN interaction with the virus does not lead to a productive infection, but to a phenomenon of viral "trapping"[140]. This process allows the virus to remain infectious for prolonged periods of time, and consequently may promote transport of infectious particles to the lymph nodes[141]. Although this molecule is pivotal for viral dissemination, it may not play a role in mucosal infection as it is not expressed on Langerhans cells within the vaginal walls[142]. Thus it is more likely that infection of immature DCs via CCR5 may be the primary source of the earliest events in HIV infection within the mucosa[137]. Additionally, recent studies have demonstrated that Tat can directly induce chemokine expression in

DCs, leading to substantial T cell recruitment, further supporting the role of DCs in early events of viral dissemination following infection[143].

DC numbers are reduced in a variety of tissues during HIV infection[144]. In addition, these cells appear to be compromised in their APC function, by inadequately priming T cells, and inducing impaired humoral responses[144]. Clear reductions in MHC class II expression on the surface of this cell subset has been observed, and may account for the inefficient activation and priming of CD4+ T cells during the course of infection[145]. Whether HIV infection directly disrupts cytokine production by DCs[146] or leads to reduced expression of CD80/CD86[131], is still unclear. Additionally, it is known that secretion of IL-12, a critical inducer of Th1 responses and activator of both NK cells and CTLs, is severely compromised in HIV infected DCs[147].

1.9.4 Lymphoid Tissue:

The first cells to come in contact with HIV or SIV during mucosal infection appear to be monocytes/macrophages and Langerhans' cells[148,149], as mentioned above. In the context of vaginal infection in the SIV model, viral replication is detectable in the iliac lymph nodes within a few days[148], and within tissues within 2 weeks of infection[149]. During rectal infection, uninfected DCs in the rectal mucosa allow for the transfer of the virus to CD4+ T cells in lymph nodes(LN) via binding to DC-SIGN[139]. During early infection, viral particles localize to the surface of follicular dendritic cells (FDCs), rather than in lymphocytes within the LNs[150]. Vigorous viral replication occurs in LNs[151] at this early time in the diseaes. Typically, LNs swell due to extensive proliferation of HIV-specific lymphocytes and due to the significant influx of lymphocytes to germinal centers (B cell areas) and paracortical areas (T cell areas)[152], making lymphadenopathy the

second most common symptom during the acute retroviral syndrome[153]. Although as many as ~1-2% of HIV infected lymphocytes are detectable in the circulation, it appears that the targets for novel infection are predominantly located within the LN tissue compartments. Throughout the asymptomatic stage of disease, increasing numbers of resting latently infected CD4+ T cells localize within LN mantle regions[154]. Gut associated lymphoid tissues, containing the majority of activated CD4+ T cells is another major target for viral infection in untreated disease[155]. Increased infections of cells within the lymphoid tissues appear to be the predominant contributor to changes in the level of free virus during infection[156].

Following initiation of HAART the destruction of lymphoid architecture caused by uncontrolled HIV infection is resolved, to a certain extent[149]. Immediately following reduction in viral load, there is an observable increase in lymphocyte numbers in the circulation. This increase is mainly due to the redistribution of memory cells from LN rather than to newly emerging naïve cells[157,158]. Following a period of therapy, naïve T cells begin to emerge from the thymus and repopulate the circulation and lymphoid tissues[159].

As untreated infection progresses, lymph node architecture is severely disrupted as CD4+ T cells and FDCs are systematically destroyed[160]. Intense hyperplasia is associated with developing disease, with characteristic depletion of LN CD4+ T cells and FDCs during end stage disease[161]. Recent work in the SIV model suggests that the level of peak viremia is associated with the degree of lymphoid atrophy[162]. Monkeys displaying high peak viremia during primary infection displayed pronounced lymphoid destruction as compared with monkeys manifesting lower peak viremia.

1.9.5 The Humoral Response

Antibodies to HIV are detectable within 2 to 3 weeks after infection and are directed at a variety of viral gene products. Despite the abundance of antibodies following infection, a large proportion may not have antiviral effects[163-166]. Some antibodies directed towards the Env gp120 glycoprotein can block viral entry and thus neutralize viral infection[167-169]. A subset of these antibodies are directed at the V3 loop, hyper-variable region, or to the "neutralization face" on gp120 that overlaps the CD4 binding site[170]. Those antibodies that bind the V3 loop are more effective at binding autologous strains than laboratory strains, and better at neutralizing recent autologous isolates. It is likely, that HIV may escape antibody control early in infection due to its high mutation rate. Regions targeted by antibodies may change early in infection and evolve to escape antibody induced pressure[163]. Similarly, heavy glycosylation of gp120 may mask regions important for antibody recognition[171].

Several observations suggest that this arm of the immune response may not be effective at controlling viral infection. 1) Few LTNPs[172] and EUs[115,173] have neutralizing antibodies. 2) Reduction of plasma viremia precedes the induction of antibodies in HIV primary infection (PI) [174,175]. 3) There is no clear correlation between antibody titer and protection[106]. 4) Although passive administration of neutralizing polyclonal or monoclonal antibodies can protect macaques challenged with SIV, extremely high concentrations of these antibodies are required to induce protective immunity to primary isolates[176]. Yet, in support of a role for neutralizing antibodies in control of HIV infection, a reduced risk of vertical transmission has been observed in infants who received neutralizing maternal antibodies[165].

Other types of antibodies may play a role in HIV infection[16]. These include antibodies associated with opsonization of infected cells, as well as those involved in antibody-dependent cell mediated cytolysis (ADCC). Presence of antibodies responsible for ADCC has been shown to be temporally correlated with reduction in viral load in PI[177].

Several recent studies have begun to delineate factors that may be responsible for the ineffectiveness of the humoral arm of the immune response in controlling HIV infection. Although neutralizing antibodies are present in the early stages of the antiviral immune response, these antibodies are incapable of neutralizing dominant viral isolates[16]. Some changes in the viral genetic sequence interfere with antibody recognition due to escape or conformational changes in the structure of gp120[164,166], as mentioned above. Yet new data reveals that other mutations may in fact lead to the rearrangement of sugar moieties on the molecule[178]. Given that there are 25 glycosylation sites on the gp120 molecule, the redistribution of sugars on the HIV glycoprotein can dramatically affect antibody access to their cognate epitopes. Kolchinsky et al. demonstrated the impact of sugar molecule rearrangement, as removal of certain glycosylation sites rendered previously insensitive viral isolates sensitive to neutralizing antibodies[179].

1.9.6 The Cell Mediated Immune Responses:

CD4 T cells can be separated into Th1 and Th2 subset depending on their cytokine secretion profiles[55]. Th1 cells are distinguished from the Th2 subtype as they secrete IL-2, IFN-γ, TNF-α, which are integral to establishing a strong cell mediated immune response. In contrast, Th2 cells secrete IL-4, 5, 6, 10 and 13, which preferentially drive a humoral response[55,56]. Differentiation of precursor Th0 CD4+ T

cells into one of the two subsets appears to be greatly influenced by the antigen[180] or the cytokines present in the milieu at the time of immune activation.

Cytokine profiles during the course of HIV infection, suggest that HIV infected subjects who exhibit Th1 type responses during the asymptomatic stage of disease control their virus more effectively, and development of a predominant Th2 response is associated with progression during the symptomatic stage of infection[181]. Concurrent loss of CTL effector function, during the symptomatic phase of the disease, is associated with the shift towards a Th2 response[182].

1.9.6.1 T cell activation

Naive T cells normally circulate from one lymphoid compartment to another via blood or lymph[183]. Entry into the spleen is non-specific, while entry into lymph nodes is dependent on the expression of CD62L, an adhesion molecule that allows cells to penetrate the high endothelial venules (HEV), and CCR7, a lymphoid organ homing receptor. Naive T cells, which are CD62L+ and CCR7+ can enter LNs[184]. Initiation of an immune response requires transport of foreign antigens to the lymphoid organs. As APCs enter the T cell areas of LNs, they begin to interact with circulating naive T cells. T cells that bind specifically form a tight immunological synapse with the APC within minutes[185]. Synapse formation leads to the accumulation of a number of cell surface and intracellular proteins involved in efficient cell signaling. Cell surface molecules that may be essential in synapse stabilization or T cell activation include CD28 (important for IL-2 induction), LFA-1, CD40L, ICOS, OX40, CD2, and CD27[186]. T cells that do not bind specifically exit the lymph node. Thus antigen specific T cells are trapped and become immobilized[187]. Following immunological synapse formation, naïve T cells proliferate

and differentiate into effector cells that can play a role in elimination of the pathogen. Activated effector cells are then excluded from the lymphoid organs as they down-regulate their CD62L and CCR7 surface molecules[188]. Following activation, clonal expansion increases the size of the pool of antigen specific effector T cells which are needed to clear or control the pathogen. Non-lymphoid homing and death may account for the exponential contraction of the clonal pool of T cells. A number of effector cells localize to the spleen or to the gut, while the greater proportion die within 5-7 days following activation[189]. Only a small proportion of the effector cells survive to become long-lived memory T cell[184,189].

IFN-γ plays an integral role in the contraction of activated CD8+ T cells, as IFN-γ-/- mice exhibit a poor contraction within the antigen specific effector pool following CD8+ T cell activation[190]. CD4 T cell contraction involves a negative signal from CTLA-4[191], followed by cytokine insensitivity, and finally the interaction of Fas/FasL[192]. Otherwise, both CD4 and CD8 T cells die if they do not receive survival signals from protective cytokines[193].

1.9.6.2 Memory Markers

Differentiation of T cells can be monitored via the expression of surface molecules that are essential for cell circulation and activity at particular stages of naïve, memory and effector T cell development. These molecules allow different subsets of cells to circulate/home through different immune system compartments and to respond to antigen stimulation differently[184]. Several markers are critical for classifying the differentiation status of cells. CD45 (a protein tyrosine phosphatase component of the TCR signaling pathway, acting as a positive regulator of Src family protein tyrosine

kinases (PTKs) such as Lck), CCR7 , CD62L, CD28 (the T cell receptor that binds B7 molecules on APC that is required for T cell activation), CD27 (traf-linked tumor necrosis factor receptor family member costimulatory molecule), perforin, and α-TRECs (T cell receptor excision circles) are a few markers that are integral to our understanding of T cell maturation (Figure 11).

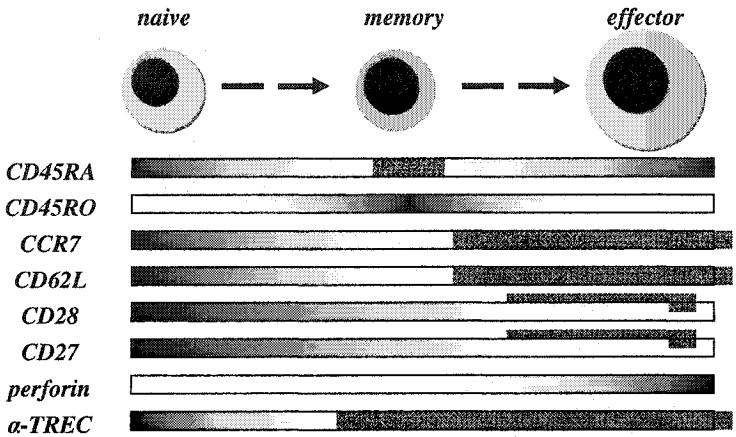

Figure 11. T cell memory markers.

Two isoforms of the CD45 molecule are presented on the surface of T cells at differential stages of maturation. The RA, or the long isoform, is predominantly present on the surface of naïve and end stage effector T cells[194]. Conversely, the RO or short isoform, is found on the surface of memory cell subsets. Both CD27 and CD28 are expressed on the surface of T cells throughout maturation, and are only lost when cells reach the end stage effector phase[195]. CCR7 and CD62L are predominantly expressed on the surface of naïve T cells; the loss of these two molecules marks mature memory cells[184]. α-TRECs are the result of successful rearrangement of the TCR α-chain. Given that gene rearrangement leads to the excision of pieces of germline genetic material, identification of α-TREC+ cells is an excellent marker for naïve T cells that have recently emigrated from the thymus[196]. Finally, perforin expression is an important marker of end

stage cytolytic effector cells[195]. All together, naïve cells are characterized by the expression of CD45RA+CD27+CD28+CCR7+CD62L+TREC+Perforin-, memory cells express CD45RO+CD27+CD28+CCR7-CD62L-TREC-Perforin-, and end stage effectors express CD45RA+CD27-CD28-CCR7-CD62L-TREC-Perforin+.

1.9.6.3 Development of T cell Memory

The mechanism by which cells are selected to become long-lived memory cells is still unclear. For example, it is still unknown whether cells are specifically selected to become memory cells based on their TCR interaction, or if this is a passive process. The theory behind the passive memory pool formation is that effector cells produced later in infection may be retained to populate the memory pool, while cells that appear earlier receive a death signal[197]. Thus, cells that are recruited earlier to the site of infection, that encounter large doses of the antigen lose the ability to express CCR7 and become polarized effector cells. In contrast, cells that appear later at the site of infection may avoid cytokine polarization and retain CCR7 expression and thus establish the pool of central memory cells.

It is clear that effector T cells may give rise to memory cells and vice versa, this pathway has been described in other viral infection models such as LCMV and CMV[198]. A striking defect in the generation of HIV specific memory subsets has been identified in the context of HIV infection, that could potentially explain why viral control is lost in HIV infection as disease progresses[199]. While significant numbers of end stage CMV-specific effector cells (CD45RA+CCR7-CD62L-) are present in CMV/HIV coinfected subjects, there is a dramatic deficit in this subset among HIV-specific cells. HIV specific cells, observed via tetramer staining, accumulate in a memory (pre-effector) compartment

(CD45R0+CCR7-CD62L-), which are weakly cytolytic[199]. Several groups have hypothesized that the loss of HIV-specific CD4+ T cells during HIV acute infection/early disease (AIED) may be responsible for delivering fewer maturation signals to memory cells resulting in skewing in the distribution of HIV-specific memory subsets such that pre-effector cell types accumulate[194,195].

1.9.6.4 Cytotoxic T Lymphocytes:

HIV specific CD8+ T cells comprise the effector arm of the immune response to invading viral infections. CTLs appear to be integral for the clearance of initial plasma viral loads[174,200], and the maintenance of viral set points in HIV infection[175]. When CD8+ CTLs recognize foreign antigens they are selectively activated, proliferate and become effector cells. The critical need for these cells was shown when depletion of these cells in SIV infected macaques led to uncontrolled replication of virus in either the primary phase or the chronic phase of infection, and subsequent rapid progression to disease[201,202]. Strong and broad HIV-specific CTL responses have been observed in LTNP[172,203,204], who control their viral loads spontaneously. Such responses have also been seen in EUs[113,115,116,173]. The presence of sustained HIV specific CTL responses in both these groups of patients implicates these responses in protection against infection and disease progression. Furthermore, broader responses are associated with better clinical prognosis[105] and loss or weakened CTL responses correlate with disease progression in late stage disease[182,205].

HIV specific CD8+ T cells can kill infected cells either through direct killing, via the release of perforin and granzymes, or through the release of anti-viral factors[206]. Interferon-γ (IFN-γ) is secreted at all stages of infection, and may be protective due to its

inhibitory effect on viral replication[207]. CTLs also appear to secrete varying degrees of tumor necrosis factor-α (TNF-α). This cytokine seems to have pleiotropic effects which can both activate more vigorous immune responses as well as up regulate viral replication[208]. CD8+ T cells may reduce viral replication by secretion of β chemokines such as MIP-1α, MIP-1β, or RANTES[209,210], which block viral entry by binding the co-receptor for HIV entry, or via the release of CAFs (CD8+ T cell antiviral factor), which suppress viral replication by blocking LTR-mediated transcription[211].

Despite the wealth of evidence supporting a role for CTL activity in controlling HIV infection these antiviral responses are unable to clear the infection. The reasons for this may be related to HIV-specific CTLs being functionally defective. As few as 15% of HIV-specific versus 50% of CMV specific CTLs stain for perforin and kill efficiently[195]. HIV specific cells appear to be compromised in their ability to develop into fully mature effector killer cells, which could contribute to their inability to effectively lyse their targets, as mentioned above[199].

1.9.6.5 CD4+ help

CD4+ T cells secrete IL-2 once activated, which can stimulate both the cell-mediated immune response, as well as the humoral immune response. Additionally, a subset of these cells is able to directly kill their targets through the secretion of granzyme and perforin[194]. During the course of infection, HIV specific CD4+ T cells become activated. Douek et al. have shown that HIV-specific CD4+ T cells are more susceptible to HIV infection than cells of other specificities and that naïve CD4+ T cell are more susceptible to infection than memory cells[212]. The exquisite sensitivity of naïve HIV-specific cells to infection is probably related to their activation upon HIV encounter,

which renders them permissive for HIV replication and the fact that they do not secrete the antiviral factor, IFN-γ, immediately upon activation as do memory cells[212]. Skewed maturation of the CD4+ T cell memory compartment, in addition to reduced expression and secretion of IL-2, but not IFN-γ, is associated with progression to disease[213]. Whereas the potential to produce IL-2 was sustained in the CMV specific CD4+ T cells and in the HIV specific CD4+ T cells of LTNPs, IL-2 secretion was not induced in HIV-specific CD4+ T cells from chronically infected HIV patients[213]. In LTNPs the frequency of CD4+IFNγ+IL-2+ CD45RA+ CCR7- cells, a distinct population of phenotypically mature HIV-specific CD4+ effector T cells, was inversely correlated to the viral load.

The impact of a strong CD4+ response on the development and persistence of CD8+ responses is a currently an intense area of investigation. Several recent reports have shown, using small animal models, the importance of CD4+ responses for the generation of memory CD8+ cells[214-217]. In HIV infection virus-specific CD4+ T cell function, as measured by proliferation to HIV proteins, becomes compromised early in infection in all but LTNP who are able to spontaneously maintain low viral load levels[218]. This could be one of the factors that accounts for persistence of the infection as only suboptimal T cell help is available for establishment of memory and an effective CTL response. As mentioned previously, lack of CD4+ help in HIV infection, as well as in other viral infections, has been speculated to be responsible for important defects in the cell mediated immunity. Absence of T cell help leads to inefficient priming of CD8+ T cells that express less IFN-γ[195]; skewing of effector maturation as immature CTLs accumulate during progressive HIV infection[199]; and loss of essential CD8+ T cell responses[219].

CD4+ T cell function deteriorates as infection progresses. Both IL-2 production and proliferation are lost first to HIV antigens, then to recall antigen, next to allo-antigen, and finally to mitogens[220]. Patients who have responses to all these stimuli appear to have a better clinical outcome, whereas those who have lost the ability to respond to these antigens progress more rapidly. Both absolute CD4+ T cell numbers and their ability to proliferate in response to p24 antigen stimulation are inversely correlated with viral load[218]. Additionally, patients with progressive HIV infection have fewer IFN-γ secreting CD4+ T cells than patients who are in HIV PI or are LTNPs[221]. Additionally, T helper activity correlates well with Gag specific CTL in that they are both inversely related to plasma viral load[219]. T helper activity as measured by proliferation is associated with the presence of a number of CTL precursors. Although CTLs are observed in the absence of T helper activity in chronic infection, these responses do not seem effective in long term control of viral replication[219]. Moreover, treatment of chronic infection does not seem to be able to reestablish or allow new HIV specific CD4 responses to develop[218,222,223], while treatment of primary infection seems to overcome this problem via preservation of these essential responses before deletion[218,224].

1.9.7 Other Viral Infections:

Although infections such as influenza can be cleared efficiently by a healthy immune system, a number of viral infections exist that are not cleared, but rather are controlled indefinitely by the immune system. EBV and CMV infections in humans and LCMV infection in mice are a few examples of well-studied models of controlled chronic infections[163]. Characterization of the mechanisms by which the immune system responds and then controls these infections allows us to unravel the mysteries behind

immunological control of persistent viral infections. The immune system is vital to the control of these infections, as they generally only cause serious disease in populations that are immunosuppressed[163]. Understanding the process of long-term viral suppression in these viral models may aid in understanding why control of HIV is ineffective, and why disease progresses in 95% of infected patients.

Murine LCMV infection is characterized by an intense activation and expansion of CD8+ T cells during acute infection, which is temporally correlated with a reduction in plasma viremia[163]. The virus-specific T cell pool then contracts although an antigen-specific memory response persists thereafter. T cell subpopulation depletion experiments in this LCMV infection model showed that CD8+ T cells played an integral role in viral clearance and that CD4+ T cells were involved in maintenance of effective CD8+ T cell responses[219,225]. Mice that lacked CD4+ T cells were able to induce strong CD8+ T cell responses during the acute stage of infection, but the virus was poorly controlled in the chronic phase of infection[219]. When CD4+ T cells were removed during chronic infection, CD8+ T cells were still able to respond quickly and effectively. In contrast when T cell help was impaired during the priming phase in the acute response to the virus, CTLs were unable to respond effectively to a second challenge even if the CD4+ T cells were present at this time[209-212]. Thus it is clear that functional antigen-specific CD4+ T cell responses must be present during a primary immune response to establish effective memory responses that will maintain long term control of the virus.

Observations of this kind made in small animal models may have important implications for HIV pathogenesis. HIV-specific CD4+ T cell loss and anergy that occurs early in infection[212] may lead to the suboptimal priming of the virus specific CD8+ T cell

compartment. By analogy with the murine LCMV infection model, the outcome would be ineffective establishment of a virus specific memory pool and the production of ineffective responses to the virus. HIV specific CD4+ helper responses appear to be present during acute infection and may be able to induce effective CTL responses at this time. Given the gradual depletion of HIV specific CD4+ T cells, newly evolving CTL responses may lack the CD4+ help during the priming phase, as mentioned earlier.

Up to 80% of adults harbor a persistent EBV infection that is asymptomatic[163]. Acute EBV infection is typically marked by the induction of a massive proliferation of virus specific cells[226]. Reduction in EBV replication coincides with emerging EBV specific CTL activity[227]. Moreover, transfer of EBV specific T cells to immunosuppressed patients with B cell lymphomas can lead to protection from disease progression[227]. Early immune responses to EBV are typically monoclonal or restricted to a few clones[228].

Approximately 70% of adults harbor a persistent and asymptomatic CMV infection. CMV reactivation causing symptoms can occur in populations of patients undergoing immunosuppressive therapy following bone marrow transplantation[163]. CMV viral replication and pathogenesis became prevalent in a significant population of end stage AIDS patients, due to the inability of the immune system to control the virus in severely immunosuppressed patients at this stage of the disease. Transplant patients who do not have CMV specific helper responses following bone marrow reconstitution do not develop CMV specific CTLs capable of controlling the virus. Yet, patients with intact CMV specific help maintained effective CTL responses indefinitely[229]. This data

supports the role of CMV specific helper cells in direction of an effective immune response, including CMV specific CTL, against the virus.

1.10 Primary HIV Infection (PI):

Only a few years after the identification of the causative agent of AIDS, HIV PI was documented. Intense study of HIV PI was driven by several emerging concepts about HIV. First, viral loads were found to be 100-1000 fold higher in PI than in chronic infection (figure 12)[230-232]. This spike in viral load led to a higher risk of transmission at this stage of disease[232]. Based on these observations investigators speculated that identification of individuals at this stage of disease could reduce the likelihood of their continuing high risk behavior that could lead to spread of the infection. The second concept related to the realization that prolonged effective highly active anti-retroviral therapy (HAART) would likely not be able to eradicate HIV from those infected[233-236]. Researchers hypothesized that reducing viral load during acute infection could reduce viral dissemination and the seeding of stable viral reservoirs[150,237]. Third, one of the most profound defects in the immune system caused by HIV infection is the loss of HIV-specific CD4+ responses[218]. Researchers in the field observed that initiating HAART early in infection rescued the ability of CD4+ T cells to proliferate to HIV proteins[238]. This led to the hypothesis that treatment initiated early enough during infection could prevent these cells from being infected and lost making them available to help virus-specific CD8+ T cells. Preservation of these responses may be the key to the effective containment of the virus in the setting of immunotherapeutic or vaccination strategies[239,240]. Finally, transmission of HIV infection appears to be relatively homogenous with one or few viral strains being transmitted; the virus diversifies soon

after infection[241-243]. Initiation of therapy in acute infection may limit the development of variants, and may aid the immune system to hold the viral load in check. For the reasons outlined above the identification and study of patients in early HIV infection had the potential to reveal much about HIV pathogenesis and to generate many possible benefits for HIV infected patients with respect to diagnosis and effective therapy guidelines.

1.10.1 Transmission:

The HIV transmission rate per sexual contact between an HIV infected male and an uninfected female partner is around 0.003[244], and about half this rate for transmission between an infected female and an uninfected male[245]. These low transmission rates seemed to be inconsistent with the higher rates at which individuals were being infected in some populations. A number of epidemiologists began to speculate that certain individuals may have been more likely to transmit the virus than others[230,232]. Based on a number of behavioral studies, researchers hypothesized that elevated rates of transmission occurred in newly infected individuals who engaged in high risk behavior. These acutely infected individuals had high viral loads, and would therefore present an increased risk to their partners[231]. A study conducted aiming to assess the likeliness of transmission in a cohort of serodiscordant couples over a 30 month period showed that HIV positive partners with viral loads in excess of 4.9 $\log_{10}$ were more likely to infect their partners than partners with a viral load of 4.6 $\log_{10}$[246]. HIV+ partners with viral loads under 3.2 $\log_{10}$ did not transmit the virus to their partners.

The biological explanation for the increased risk of transmission in PI lies in the association between the level of viremia in blood and in semen at this time, although this association is not seen in all individuals[230,231]. Epidemiological studies documented the

role of promiscuity in increased risk of transmission, so that individuals with more partners, and a higher incidence of sexually transmitted diseases were at higher risk of transmission, possibly due to increased numbers of lesions and inflammation associated with these lesions[231]. These two factors that increase risk of transmission are not mutually exclusive and may be interrelated as individuals who habitually lead lifestyles that include high-risk behavior may have higher incidences of sexually transmitted diseases (STDs), be promiscuous, and potentially engage in sexual activity with more partners during PI leading to higher rates of transmission.

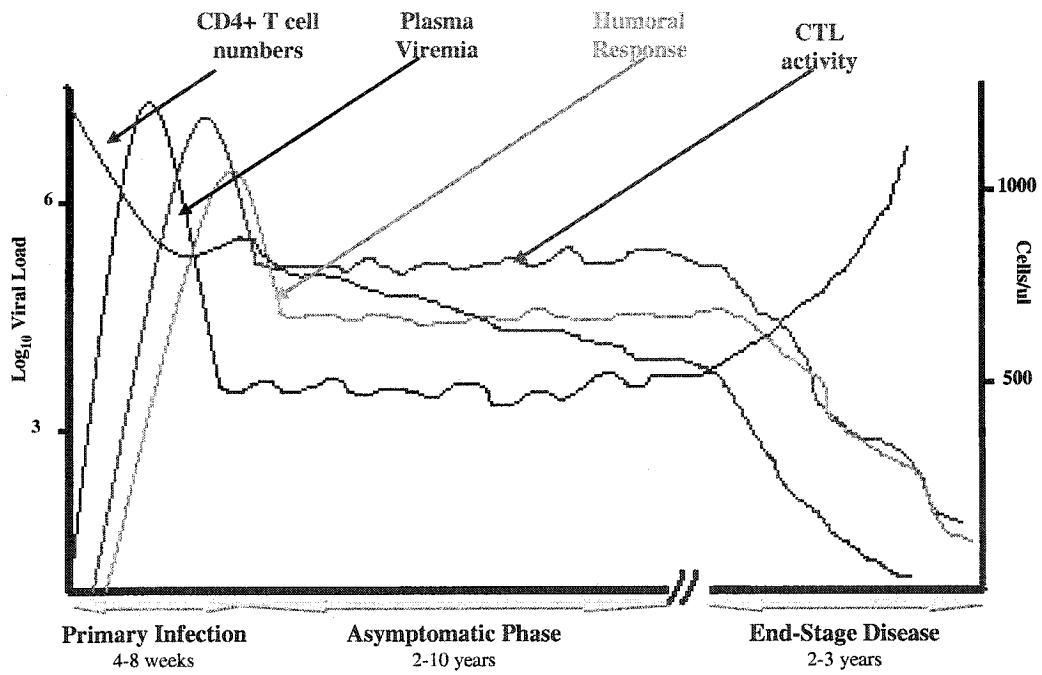

Figure 12. HIV clinical course

1.10.2 Viral Reservoirs:

With the advent of HAART, it was hoped that HIV could eventually be eradicated from infected individuals as virally infected cell and plasma viral pools would decay with

time on therapy. Treatment with HAART was able to reduce plasma viral loads to levels undetectable by available assays (less then 20-500 copies of RNA/mL) in many individuals adhering to their drug regimens. However, the discovery of several viral reservoirs with long-lived kinetics led to the realization that it was unlikely that years of effective therapy would be able to eliminate the virus[150,233,236,247].

HIV PI is characterized by high levels of viral replication with more than 10^6 copies of the virus/mL of plasma(figure 12)[248,249]. Viral reservoirs are apparently seeded in the early burst of viral replication in acute infection[150]. The intense dissemination of virus at this early time, before the advent of antiviral pressure by antibodies and cytotoxic responses, may allow the virus to infect numerous sites and cell types. A number of reservoirs have been described such as immune-privileged sites (central nervous system), latently infected cells (memory CD4+ T cells), pre-integration complexes (resting CD4+ T cells) and long-lived cells such as memory T cells and macrophages[237].

After starting HAART, 3 phases of viral decay have been described[236,250]. Viral decay does not seem to depend on the stage of the disease, but on the level of viral production. Thus as productively infected cells perish, and free viral particles are eliminated by the immune system, clearance rates are consistent at all stages of disease[233,237] and are only determined by the level of viremia a patient experiences at any given time. Thus the steady state or viral set point can be taken as a good predictor of clinical outcome, as higher viral loads at set point reflect higher viral production than in patients with lower viral set points who effectively have lower rates of viral production or higher degrees of clearance[251].

The first decay phase is rapid with a $T_{1/2}$ of approximately 2 days and reflects the clearance of free virions and productively infected cells that produce most of the plasma viremia[157]. The second phase of decay has a $T_{1/2}$ of 1-4 weeks[250]. Virus eliminated in this phase comes from productively infected cellular reservoirs such as those eliminated with time by the immune response. Based on mathematical models, Perelson et al. hypothesized that it would take 2-3 years of effective HAART therapy to eliminate this reservoir of virus, initially believed to lead to the complete elimination of the viral infection[157]. The third phase of decay is that of virus in undetectable reservoirs that are resistant to eradication by HAART[233]. This reservoir is the source of reemerging virus after therapy is withdrawn, leading to *de novo* reseeding of the tissues[252]. This reservoir is exceedingly stable and contributes minimally to the total viral production in untreated individuals as it goes unnoticed by the immune surveillance systems. CD4+ memory T cells have been implicated as the source of this reservoir[253]. The biology of these cells dictates that they persist for long periods of time in a resting state and only become activated once again after encounter with their cognate antigen[254]. Once reactivated, they can give rise to large numbers of clones all containing the proviral sequence. Three groups have successfully demonstrated the role of these memory CD4+ T cells as long-lived viral reservoirs, by culturing replicating virus from such cells isolated from patients after 2.5 years of successful HAART therapy[233,255,256]. Given that resting memory cells are quiescent, cellular proteins required for viral replication are not produced and the virus remains latent. In this manner HIV in resting cells are resistant to antiviral therapy, which targets steps of the active viral life cycle[257]. Mathematical modeling suggests that it would take 73 years to eradicate all viral reservoirs with aggressive HAART[258].

Recently, longitudinal studies monitoring reservoir decay over 6-7 years of effective HAART showed no significant decay over time in resting T cells[254]. Because prolonged HAART is unable to eradicate viral infection new recommendations have been proposed for treating HIV infected persons that delay initiation of aggressive therapy in order to diminish long-term exposure HAART with its associated side effects and potential for developing drug resistant variants[258].

1.10.3 CTL Responses in PI:

Cytotoxic T cell responses appear early after infection, and their induction is temporally correlated with the reduction of plasma viremia (figure 12)[174,200], as mentioned earlier. As many as 10% of circulating CD8+ cells, which peak soon after plasma viral load begins to fall, can be specific for HIV peptides during the acute phase of disease as seen by staining with tetramer reagents that mark HIV-specific cells[259]. It appears that some HIV+ subjects in acute infection have monoclonal responses to the virus[228], and viral diversification may contribute to evolving HIV specific responses with time from infection[241,242,260]. Although levels of viremia fall in subjects with oligoclonal and monoclonal TCR variable β chain expansions[261,262] and CTL responses[243,262] to the virus, monoclonal responses appear to predict a poor disease course prognosis[263]. Therefore early patterns of HIV specific immune responses may determine the long term course of the disease[264].

1.10.4 HIV pathogenesis

Strains of HIV isolated from early infection tend to be non-syncitium inducing (NSI); macrophage tropic viral isolates that use CCR5 as their co-receptor for entry into

target cells[16]. Infection of macrophages may confer the virus with a survival advantage as these infected cells are part of a long-lived stable viral reservoir[236]. As disease progresses and viral mutations accumulate, a shift in the phenotype to syncitium inducing (SI) occurs. SI viruses can grow in tissue culture adapted T cell lines, i.e. become T-cell tropic and can use CXCR4 as an entry co-receptor. CXCR4 has a wider distribution on CD4+ T cells than CCR5[16].

Kinetic studies on $CD4^+$ T cell turnover after initiating HAART in treatment of naive individuals shows that accelerated destruction and replacement of $CD4^+$ T cells takes place continuously, from the time of initial infection (figure 12)[265,266]. This phenomenon may be responsible for the eventual exhaustion and nearly complete disappearance of $CD4^+$ T cells from untreated infected individuals (CD4 sink model) (figure 12)[250,265,266]. Several other mechanisms have been proposed to explain the numerical and functional depletion of $CD4^+$ T cells: 1) Direct cell-killing through antigen-specific activation of $CD4^+$ T cells by the virus[266,267]; 2) death of infected cells resulting from the accumulation of viral genetic material and/or the presence of virally encoded toxic proteins[268]; 3) formation of syncytia[269]; 4) induction of apoptosis and/or of an anergic state in both infected and non-infected bystander cells[270]; 5) direct ADCC or HIV-specific T lymphocyte cytolysis of infected or bystander cells[175,271]; and 6) delivery of an apoptotic signal through deviant TCR/CD4 engagement by gp120 and MHC class II molecules present on the surface of the virion[118].

1.10.5 Diagnosing PI:

Acute infection with HIV is frequently associated with a self-limiting mononucleosis like illness. The acute seroconversion syndrome can include fever,

pharyngitis, lymphadenopathy, rash, headache, gastrointestinal problems, genital and oral ulcerations[249]. Sixty-90% of HIV infected subjects report having had one or more symptoms of acute infection[153,249,272]. The time interval between the virus transmission event and the start of an acute seroconversion syndrome varies anywhere from a few days to a few weeks[272-274]. Its duration generally lasts 7-10 days, infrequently lasting more than 2 weeks[273]. Of persons who exhibit symptoms of an acute seroconversion syndrome a significant fraction seek medical attention during this early period of infection. The symptoms of this syndrome are non-specific and are often not attributed to a new HIV infection[274].

A number of hematological changes often accompany PI. Lymphopenia and thrombocytopenia are present during the first week of HIV infection. Soon after these changes, the number of lymphocytes increases dramatically with a characteristic rise in CD8+ cells while CD4+ cell numbers decrease[16]. An inversion in the ratio of CD4:CD8 is a hallmark of HIV infection.

It takes approximately 3-4 weeks from infection (range 2-12 weeks) to develop antibodies to p24 (figure 12)[275-277]. As the enzyme-linked immunosorbant assay (ELISA) test, detecting p24 antibody (EIA), has a high rate of false positives, all positive results are confirmed by Western blotting. A positive confirmatory western blot is defined as having at least a positive signal for a minimum of 3 separate HIV proteins. If both the standard HIV EIA and the confirmatory western blot are positive the plasma donor is said to be seropositive and diagnosed with HIV infection. A Western blot with less than 3 bands is considered an indeterminate result, occurring frequently before "seroconversion" and can be used to identify potential new infections. Several additional tests are available

for the detection of recent infections that are capable of detecting the virus prior to the time at which the standard diagnostic test can detect the infection. A positive result in an ELISA test detecting p24 antigen in plasma and a positive result in a viral load test detecting HIV RNA in plasma by RT-PCR and a positive result by PCR for proviral DNA will occur before the standard HIV enzyme immunoassay (EIA) detecting antibodies to HIV p24 will be positive[278].

Choosing the right test involves consideration of the induction kinetics and concentration of the parameter being tested during the course of infection. Viral RNA can be detected within the first 2 weeks using the highly sensitive RT-PCR method[278]; antigen, although transient, can appear as early as 2 weeks after infection and last 3-5 months[153]; whereas antibodies, which develop later in the course of infection, are detectable in a standard EIA only 4-6 weeks post infection[275](figure 12). The use of plasma RNA and proviral DNA as diagnostic markers of disease have proven to be problematic due to the cost, technical training required to run a test, and the probability of a false positive result. Daar et al. found that despite the fact that no distinct set of symptoms were associated with acute HIV infection, simultaneously testing all cases of mononucleosis-like illness for antibody to HIV p24, HIV RNA and HIV p24 antigen did detect a significant number of recently HIV infected patients that were overlooked by using the standard EIA assay alone[279]. Yet the cost of the HIV RNA assay, pre- and post-counseling, to advise patients of potential false-positive results led to the conclusion that although this test may be important, it is not necessarily cost and time effective. As p24 antigen testing detects 90% of all HIV positive patients that are missed by the p24 antibody test alone, it has been recommended that these two tests be done in suspected

cases of new infections in order to provide treating physicians a measure of confidence in their diagnosis of recent HIV infection[279].

1.11 Chronic Infection:

Resolution of PI leads to a prolonged period (2-10 years) of asymptomatic disease (figure 12)[280]. In contrast to other viral infections, where CD8+ T cells contract as viral replication falls, expanded oligoclonal HIV-specific cells persist at relatively high frequencies of 1-2% of circulating CD8+ T cells[281]. Untreated chronic infection represents a dynamic period during which continued viral replication is maintained at a viral set point via the persistence of the host immune response[248]. These cells are likely sustained due to antigen stimulation, as initiation of therapy that reduces viral replication, leading to a loss of antigen, results in a decline in antigen-specific CD8+ T cell numbers[282-284]. The use of tetramer reagents to detect HIV-specific cells confirms that such cells are detected, although less frequently than at earlier times in infection, during end stage disease (figure 12)[285]. Despite their numbers in chronic infection, HIV specific cells appear to have less effector functions as disease progresses[195].

Increasing loss of HIV-specific CD4 numbers and function occurs with progressive disease (figure 12)[280]. HIV infected individuals lose an average of 60 CD4+ T cells a year[286]. Subjects in progressive infection appear to experience severe defects in CD4+ T cell activity over time[220,287,288], as mentioned previously.

1.12 Long Term Non-Progressors:

Approximately 5% of HIV infected individuals who have never been treated with HAART differ from others by showing few signs of disease progression despite untreated

infection for greater than 7 years[289]. Individuals belonging to this population are called LTNPs. The percentage of HIV infected individuals classified as LTNP can range from 1-25% depending on the criteria used to define inclusion in this group[289]. CD4+ T lymphocyte counts above 500 cells/uL, viral loads that are low or below detectable levels, and CD4+ lymphocyte decay curves with a slope near 0 are a few of the defining characteristics used to classify subjects as LTNP[289]. Regardless of the criteria employed to identify this population, it appears, from analyses performed on sampling distributions of HIV infected patients from large cohorts, that this group represents the tail end of a normal distribution of HIV infected persons and not a population of HIV infected subjects distinct from typical progressors[290].

In an effort to identify clinical correlates of protection, to aid in the characterization of LTNPs, a number of groups began to look for markers of non progression. No association between demographic markers and non-progression existed with respect to age, sex, mode of HIV infection, continued high risk behavior, and nutrition[291]. Similarly, no relationship was apparent between the level of antibody production and disease progression[289,290,292]. More specifically, while some studies have identified significant levels of neutralizing antibodies in LTNPs[203,293], these were not consistently present in other LTNP populations[204,289,294].

In a population of 588 men with documented dates of infection enrolled in the San Francisco Clinic Cohort, 3% progressed to AIDS within the first 3 years of infection, 51% progressed by 7 years from infection, and 69% developed AIDS 14 years after seroconversion[286]. In a parallel study, Sheppard et al. observed a strong correlation between the rate of CD4+ cell loss and the development of AIDS related illness[290].

Several studies demonstrated that only a small proportion of HIV infected subjects maintained CD4+ counts above 500 cells/ul for 7 or more years after infection, and fewer maintained a CD4+ cell count of this magnitude after 10 years[291,295]. The median time from HIV infection for an individual's CD4+ count to fall below 500 cells/ul is 1.7 years (95% confidence intervals [CI] 1.29-1.89 years)[295]. While untreated HIV disease progressors lost CD4+ T cells at a rate of approximately 60 cells/per year, LTNPs lost these cells at a rate of 6 cells/year, a rate not significantly different from that seen in HIV seronegative controls[286,290].

LTNP, differ from progressors by maintaining CD4 counts above 500 cells/ul for at least 7 years post infection and displaying less immunopathogenicity and destruction within this cell compartment of the immune system[160]. Additional markers of reduced pathogenicity in LTNP include elevated CD8+ cell counts in most but not all such individuals, higher white blood cell counts, increased platelet counts, normal CD4 T cells counts, and subsequently reduced CD4:CD8 cell ratios[286]. This population manifests reduced activation markers, such as HLA-DR and CD38 expression on their T cells, and reduced serum neopterin and β_2-microglobulin than progressors[290]. Pantaleo et al. observed reduced immunopathogenicity and lymphocyte depletion in the LNs of LTNP despite infection for at least 10 years[160]. Four of 15 LTNP LNs maintained small regular germinal centers with intact mantle zones and no evidence of follicle lysis; five subjects' nodes had large regular shaped germinal centers, with minimal follicle lysis; five additional LTNPs' had irregular unorganized LNs; where as all 18 HIV disease progressor's LNs had large irregular fused follicles with loss of mantle zones[160]. LTNP also appeared to have healthier FDC morphology than did the HIV disease progressors.

Virus isolation by co-culture assays proved to be quite difficult in LTNPs, potentially due to reduced viral load levels and/or to there being a 20- to 40-fold lower frequency of cells harboring proviral DNA in LTNPs compared to typical asymptomatic progressors[203,290,293,294,296-300].

A subpopulation of LTNP may exhibit a benign disease course because they are infected with defective HIV isolates. Learmont et al. reported that delayed progression and unrecoverable virus from 6 HIV+ subjects infected by transfusions from the same donor in Sidney, Australia 13 years earlier, was probably due to a gross defect in the Nef gene of the transmitted virus[301]. All 6 individuals, expressed different MHC class I and II alleles making it unlikely that protection was due to a common MHC restricted cellular response. All controlled viral replication to below undetectable levels. The Australian investigators hypothesized that this Nef deletion reduced the viability and fitness of the virus sufficiently to allow the hosts to independently contain their infections. HIV isolates with Nef deletions that could explain the ability of the host to contain infection were reported by 2 other groups in LTNP[294,299]. Defects in Gag p17[293,300] and Vpr[302] were also described and proposed to account for the attenuated the virulence of HIV in individuals who exhibited a benign disease course. Despite the evidence for viral attenuation in studies listed above, HIV-specific CTL activity was also observed in a number of patients[293,294], suggesting that viral replication was ongoing, albeit at undetectable levels. It was therefore proposed that these viral mutations were merely giving the host immune system an upper hand in helping it to control the infecting virus.

Infection with defective virus does not account for disease non progression in all LTNPs[203,204,292,297]. Greenough et al. observed a temporal association between HIV-

specific immune responses and increased levels of viral DNA in PBMCs[292]. Additionally, a case report published in 1996 described two unique LTNPs infected for greater than 10 years, who manifested viral loads in the range of normal progressors[303]. Despite persistent viral replication, these two individuals maintained CD4+ cells between 400-700 cells/uL. Thus these otherwise healthy subjects appeared to harbor fit viral species, yet were unable to elicit immune responses to contain their infecting virus. Cao et al. also demonstrated the presence of fit virus in a group of LTNPs who maintained undetectable viral loads[203]. The protective mechanism was associated with the refractory nature of the subjects' CD4+ T cells to infection by the fit infecting viral species. The resistance of CD4+ T cells to infection was dependent on the presence of CD8+ cells[297]. Using CD8+ depletion, Rinaldo et al. showed that cytotoxic activity against viral infection was mediated by CD8+ cells[204], an observation confirmed by others[304,305].

Multi-linear regression analyses aimed at defining the role of potential correlates of immunity in groups of LTNP identified certain host factors that were independently associated with reduced progression. Carrington et al. identified a strong association between homozygosity at the MHC class I locus with an increased risk to disease progression in five independent North American cohorts of HIV seroconverters[105], as mentioned earlier. HLA-B27 and B57, B39[111], B14, Cw14 and Cw8[306] are associated with protection, while HLA-B35[105], A29, B22, and Cw16[306] are associated with faster disease progression. Although homozygosity for a 32-base pair deletion mutation confers a remarkably reduced risk of infection, heterozygosity for this deletion mutation and several other variants of this HIV co-receptor are associated with slower disease progression[307,308].

Protection, associated with the over-represented expression of HLA-B27, in a subgroup LTNPs appears to be due to fact that up to 85% of individuals with this allele target a dominant highly conserved epitope within Gag p24 (KRWIIMGLNK)[309,310]. To gain a better understanding of the protective nature of this allele, a B27 expressing LTNP was followed over time[311]. This subject exhibited spontaneous control over viral replication below detection levels for 12 years, at which point viral titers increased exponentially with a concurrent decrease in CD4+ T cell numbers. These changes were associated with the accumulation of 3 mutations in and around the KK10 epitope[309]. The first mutation, lysine to-arginine at position 2 located within the epitope, led to reduced peptide binding to the B27 molecule. The two other mutations were located outside the epitope. These mutations appear to be critical for protein folding due to the conformational changes brought about by the lysine-to-arginine mutation within the epitope. Three of 5 additional B27 subjects manifested narrow responses directed at the same KK10 epitope associated with good control of viral replication[311]. Two of the 5 subjects studied had declining CD4 counts, a broader HIV specific response, and demonstrated reduced recognition of the KK10 epitope. Similarly, children who shared the B27 allele with their mothers' failed to recognize the KK10 epitope, while children who inherited the allele from their father's targeted this epitope in an immunodominant manner[310]. Sequence analysis revealed that mother to child transmission of the KK10 escape mutant had occurred in the mothers who had originally targeted that epitope, while mothers who did not have the B27 allele transmitted the wild type KK10 epitope, suggesting that stable transmission of viral escape mutants had occurred[310]. Thus while alleles such as B27 may appear to be associated with effective protection for a period of

time, the association of expression of this allele with targeting of a single immunodominant peptide may be quite dangerous, as once escape occurs, subjects progress very quickly given the paucity of additional immune responses directed at the virus[311].

Several additional immunological differences exist between LTNPs and progressors. LTNPs appear to exhibit greater CTL activity[160,204,293,298], to a broader range of gene products[296]; exhibit a balanced Th1/Th2 phenotype, with increased expression of IL-2, IFN-γ, IL-4, and IL-10 production[312]; possess higher proportions of end stage (RA+CCR7-CD62L-) effector cells than progressors[109,313]; have an increased proliferative capacity and produce larger quantities of perforin in response to antigen stimulation[314,315]; have more HIV-specific CD4+ IFN-γ and IL-2 secreting cells[316]; express normal levels of CD28, critical in T cell signaling[316]; and produce considerable levels of RANTES, MIP-1α and MIP-1β, all implicated in protection against the spread of viral infection as they bind the CCR5 HIV co-receptor and block HIV entry[218] than subjects in progressive infection.

Several factors including host genetics, viral fitness, and the effectiveness of CD8+ T cell antiviral immunity, and maintenance of functional HIV-specific CD4+ T cells all impact on disease progression. The identification of a subpopulation of HIV infected individuals who exhibit a certain level of resistance to the immunopathogenesis caused by this viral infection should help to decipher mechanisms that contribute to HIV disease non-progression. By understanding these mechanisms the hope is that it will be possible to transfer this knowledge to an immunotherapeutic setting with the goal of transforming HIV disease progressors into LTNP.

1.13 Viral Escape

Despite the presence of considerable HIV specific immune responses throughout infection, these responses are unable to clear the infection. HIV has developed many mechanisms to evade the immune system, these include: 1) down regulation of MHC class I molecules on the surface of infected cells by virally encoded Nef protein[24,317]; 2) sequestration of HIV to cells in immune privileged areas that are not accessible to CTLs[318]; 3) elimination of HIV-specific CD4+ T cells implicated in the generation of defective antiviral responses[218,319]; 4) up regulation of Fas ligand on infected cells[317]; and 5) mutational escape[194,310,320-322].

Viral escape has been well documented in other viral infections, such as EBV[323], Murine Leukemia virus[101], LCMV[324], and hepatitis B virus (HBV)[325]. Transgenic mice infected with high doses of LCMV failed to control the viral infection, whereas their counterparts infected with low doses of the virus cleared the virus and remained healthy[326]. On closer inspection, the virus from all mice receiving high dose LCMV carried a mutation in a critical immunodominant epitope in such a way that it could no longer be presented to the immune system. The fact that this variant epitope was poorly recognized by the immune system, was likely responsible for the lack of control of the viral infection in the mice that received the high dose antigen[324]. Another example of this phenomenon was documented in South East Asia and Papua New Guinea where HLA-A11 has a high allele frequency. In this setting EBV associated disease is more prevalent due to the transmission of a strain that has incorporated an escape mutation within an immunodominant A11 epitope that is critical for viral control[323].

The tenet that mutations within immunodominant epitopes could account for viral escape from immune recognition stems from the knowledge that HIV exhibits a remarkable amount of sequence diversity due to the low fidelity of its viral RT enzyme. Based on data from viral kinetic studies on and off antiretroviral therapy, there are about 10^9-10^{10} virions generated a day[265,266]. Given that the mutation rate is about 10^{-5} and that the genome is about 10^4 bases long, approximately 108 mutants can arise each day. Mistakes in viral RT are random and are not specific to certain regions of the virus[327-329]. Based on this it is clear that the immune system is forced to deal with every possible point mutation[330]. Therefore the chance is great that viral variants will arise in epitopes that will no longer be recognized by CTLs that recognized the wild type variant. Thus the viral variants carrying epitopes no longer under CTL pressure will have a selective advantage within the viral swarm present in any particular host.

1.13.1 Lessons from SIV studies

SIV infection of macaques allows researchers to inoculate a host with a known sequence at a given dose, through a chosen route. This model permits the identification of relatively subtle changes in the viral sequence that could contribute to a loss of CTL recognition, unhindered by confounding factors such as antiretroviral therapy, which itself exerts pressure on the natural evolution of the virus. The methodology used to study the impact of viral diversification on escape from immune control is based on a ratio of the rate of synonymous (dS) and non-synonymous (dN) mutations occurring throughout the virus and particularly in CTL epitopes[322,331]. Synonymous mutations are those that produce no amino acid changes while non-synonymous mutations produce a change in the amino acid sequence[331]. The principle relies on the following possibilities: if a dN

mutation leads to a non-functional protein, the viral variant will not be selected; if, on the other hand, a dN mutation leads to a variant that has no impact on the function of the protein but is sufficiently different to go unrecognized by the immune response, then the virus will have a selective advantage and outgrow the other viral populations being targeted by the immune response[331].

The first studies on viral escape from immune recognition were performed in 1992, where a group of Mamu A*01 expressing monkeys were monitored for sequence changes in a Gag epitope called TL9. Mamu is the macaque equivalent of the MHC, and Mamu A*01 is a MHC class I allele with a fairly high frequency in this species. Two monkeys displayed epitope variation, but changes within the epitope did not significantly exceed changes outside the epitope[332]. Closer inspection of the changes in the sequence of the optimally restricted epitope, 8 years later, revealed that the mutant epitope dissociated from the Mamu A"*01 allele more rapidly than did the wild type variant[333]. In parallel, Nef vaccinated monkeys, which were subsequently challenged with a pathogenic virus, eventually displayed a complete loss of Nef-specific CTL responses. The loss of Nef specific responses was due to the deletion of the Nef epitope in the emerging viral variants targeted by CTL[334]. Moreover, several studies have shown that regions corresponding to CTL epitopes mutate more frequently than flanking sequences[321,322,335], suggesting that CTLs are driving diversification of the viral population to eventual viral escape from immune recognition.

Escape mutations also occur in acute infection[320,321], and can potentially contribute to the mechanism by which the breadth of the immune response evolves, which could merely be a marker of the immune system catching up to viral

diversification in the face of immune pressure. Identification of a Tat epitope in acute SIV infection (Tat SL8) frequently targeted in Mamu A*01 expressing macaques showed dramatic sequence variation only in monkeys that were Mamu A*01 positive[321]. DN:dS ratio analysis suggested that this epitope was clearly evolving under strong immune pressure. It was hypothesized that the abundant expression of Tat early in the viral life cycle contributed to its antigenicity. In other words by targeting this epitope, the immune system would be able to control viral replication effectively by lysing newly infected cells before they produce new virions, thus effectively reducing viral replication during early disease[331]. On the other hand immunodominance of SL8 targeting, although not mutually exclusive from the theory that it is expressed earlier in infection, may be likely attributable to the fact that the Tat SL8 epitope may have a higher avidity than other well characterized epitopes derived from SIV[321]. Thus viral variants that escape the combined high avidity binding and early detection by the immune system have a greater survival advantage over their wild type counterparts. Thus the additive effects of early targeting and high avidity may help explain the preferential escape of SL8 responses in Mamu A*01 expressing monkeys.

1.13.2 Lessons from Human studies:

Koenig et al. attempted the adoptive transfer of autologous Nef specific CTL clones, expanded *ex vivo*, into an HIV infected patient combined with the administration of exogenous IL-2[336]. Cell transfer, rather than resulting in an anti-viral effect, resulted in increased viremia. Sequencing studies revealed the emergence of a deletion within the targeted epitope in 30% of the viral population found in the patient. A study on HLA-B8 restricted responses in progressive infection elucidated the predominance of mutations in

the HLA-B8 epitope in subjects expressing this allele in comparison to those who did not[337]. Similar findings have been reported for clusters of mutations in epitopes restricted by HLA-All[338] and B7[260].

In acute infection, Price et al. observed escape due to immune pressure in two separate acutely infected patients. The first patient exhibited a monospecific CTL response to a B44 restricted env epitope, where 100% of the viral sequences had a single mutation at an anchor residue that could no longer bind to the corresponding pocket in HLA-B44 alleles[322]. Interestingly, as this viral variant outgrew the other isolates, the CTL response diversified to recognize other epitopes within different gene products. Similarly, a second patient with a monospecific response to a Nef epitope restricted by HLA-B8, displayed evidence of emerging species of the virus with changes in the anchor residues of this Nef epitope or complete loss of the epitope from the viral sequence within a few weeks of infection[322]. In acute infection, potent CTL responses select for viral escape soon after their induction. HIV has proven to be successful in CTL immune evasion during chronic infection as well. As mentioned above, Goulder et al. demonstrated viral escape from an immunodominant HLA-B27 restricted Gag KK10 epitope in two HLA-B27+ patients, where patients displayed the same dN arginine to lysine mutation at position 2 of the epitope that prevented its binding to the HLA-B27 allele[311].

Whether an epitope escapes in early or late infection is probably determined by the location of the epitope and whether it is part of a conserved or variable region. It is likely that viral particles that escape immune control with alterations in variable regions due to single nucleotide changes are more common early in infection as these areas are more permissive to changes without affecting viral fitness. This is analogous to the

variation in viral sequence seen in the context of monotherapy with AZT[331] where single changes in the virus can confer resistance to AZT. In contrast, escape could potentially be delayed if epitopes are located in conserved regions, where a change in a conserved region, leading to escape, could be detrimental to the folding or function of the viral gene product. This is illustrated by studies conducted in SIV infected macaques relating to escape in the SIV Gag epitope CM9. A single change within the epitope is sufficient to prevent recognition of this peptide by CTL that recognize the wild type variant. This change occurs late in infection and is tightly linked to the presence of two additional dN mutations one upstream and one downstream of the epitope[333]. The changes in sequences flanking the CM9 epitope are believed to be compensatory mutations integral to the fitness of the viral isolate that escapes recognition of Gag CM9[332]. For this reason epitopes in conserved regions may take longer to escape CTL pressure as they may require more complex combinations of alterations in the gene product to produce a reasonably fit virus.

Narrow immune responses are associated with the clearance of influenza and the control of EBV infections[339,340]. In contrast, the breadth of the response to HIV may be due to a spreading of the response to multiple epitopes resulting from continuous rounds of evasion and the resulting induction of new responses[341], as referred to previously. Once escape has occurred, clones recognizing the original variant may contract due to the loss of their cognate stimulating antigen. Consequently, new variants have the potential to induce novel immunodominant responses. So too CTL epitope mutations may favor the emergence of existing subdominant responses, which may be less efficient in stimulating the immune response[342]. In chronic infection, the immune response is broad and is

directed towards a variety of epitopes, with no clear pattern of immunodominance[343,344]. By late disease multiple rounds of viral escape may result in reactivity to only sub optimally presented epitopes that do not engage the TCR sufficiently, that have lower antigenicity, or that dissociate from the MHC groove too quickly. These responses would mediate poor viral control of new variants emerging from unchecked replication.

CTL escape mutations may facilitate mother to child transmission. As mentioned earlier, Goulder et al. demonstrated faster progression in perinatal infections was associated with mother to child transmission when the child and the mother both expressed the same HLA-B27 MHC class I allele[345]. The presence of the HLA-B27 allele in the mother led to the emergence of variants in the KK10 epitope no longer recognized by a potent immunodominant CTL response frequently seen in HLA-B27 expressing subjects. Mother to child transmission of this variant together with the expression of HLA-B27 allele in the infants, resulted in the inability to mount a potent immunodominant response restricted by this allele and thus rapid disease progression. In contrast, children who inherited HLA-B27 from their fathers and virus from their mothers who did not express this allele were able to mount the immunodominant HLA-B27 restricted response to wild type KK10 epitope on the transmitted virus and effectively control viral replication.

1.14 HAART

HAART, refers to the combination of at least two of the four different classes of drugs to treat HIV infection[346]. It is the standard of care for the treatment of HIV infected patients although the recommendations on when to begin treatment have changed in the last few years. The introduction of this therapeutic strategy has altered the course of HIV

infection, having made a fatal disease into a manageable chronic infection[347]. This is primarily due to the fact that HAART can partially restore and protect immune system function and delay the development of AIDS or death from an HIV-related condition for several years. Current recommendations for the time to initiate therapy are: 1) patients who are in acute infection, 2) patients that have CD4 counts below 200 cells/ul, and 3) patients with symptomatic HIV infection including thrush or recurrent invasive bacterial infections, plus an absolute lymphocyte count of less than 1000 cells/uL[348]. Problems encountered with the use of HAART are the economic unavailability of this treatment on a global level, the inherent drug toxicities incurred with long term usage, problems with drug-drug interactions, and the potential for the selection of drug resistant mutants if 95% drug adherence is not maintained[346].

At least 2 classes of drugs are used in HAART regimens. They can include nucleoside analogue reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, and fusion inhibitors[349]. While the first two classes target the same stage of the viral life cycle, namely viral DNA transcription, protease inhibitors target viral protein maturation, and fusion inhibitors block viral particle entry (*de novo* infection) (figure 13)[350].

NRTIs inhibit the synthesis of DNA by the viral RNA-dependent DNA polymerase (RT)[350,351]. This class of drugs is structurally similar to nucleosides or nucleotides that act as normal cellular building blocks for DNA synthesis. Thus as NRTIs become phosphorylated (some are modified more extensively like ddI or less extensively like Tenofovir) within the cells, they become incorporated into newly synthesized DNA molecules as the RT cannot distinguish between the drug and the normal cellular building

blocks. Because these nucleoside/nucleotide analogues lack a critical hydroxyl group that makes further chain elongation possible, DNA transcription ends following their incorporation[16]. NNRTIs also halt viral DNA transcription by inhibiting the viral RT at an allosteric site. This class of drugs binds RT, outside of its catalytic site, and inhibits its activity[350,351]. Protease inhibitors interfere with a different enzyme involved in the HIV viral life cycle, the protease enzyme that is critical for viral protein processing and maturation[350](figure 13). This drug class binds to the enzyme, and although viral particles are still produced, these particles are no longer infective[16,349]. Finally, fusion inhibitors prevent viral entry into uninfected cells[39,352,353]. This class of drug binds tightly to the gp41 HIV gene product, blocking the essential association of this protein with the viral co-receptor located on the target cell surface. This interaction inhibits viral particle uptake, and thus infection[353,354].

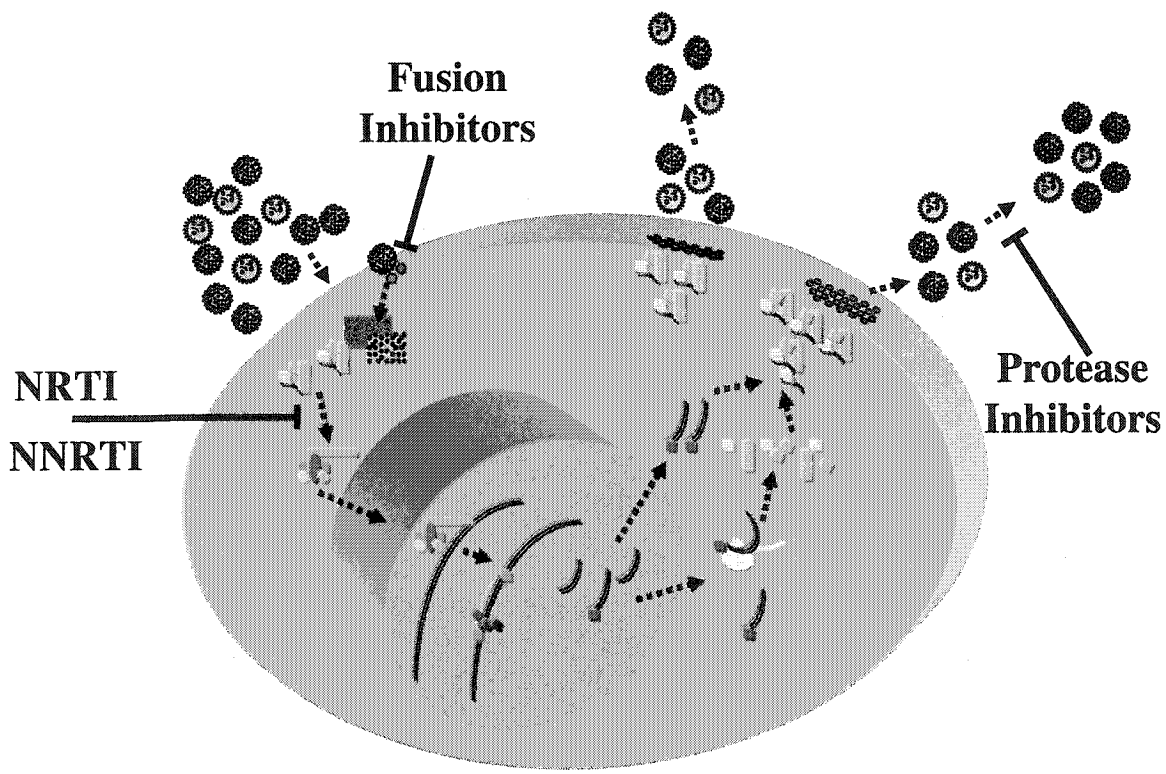

Figure 13. Points in viral life cycle at which therapeutics, included in HAART regimens, act.

One of the greatest impediments to the use of these antiretroviral drugs is HIV's innate high level of mutability, which allows the virus to incur resistance mutations that lead to unresponsiveness to the drugs[16,351,355,356]. As each therapeutic puts pressure on the target enzyme in distinct patterns at different locations, and given the high mutation rate of the virus, species of the virus that have altered drug binding sites are selected and grow out resulting in the outgrowth of drug insensitive virus. To add to the complicated nature of this problem some drugs may induce mutations that can lead to viral resistance across entire classes of drugs[16,356,357]. In order to overcome this obstacle, multiple drugs are used in combinations in order to place a greater more distributed pressure on the virus at multiple sites important in the viral life cycle. This strategy is known as HAART that aims to reduce viral replication, and thus the harmful cytopathic effects of the infection, as well as the emergence of resistant virus[348].

In order to optimize the efficacy of each drug in a particular HAART combination, complicated treatment regimens must be adhered to. Resistance to these drugs can develop quickly if too many doses are missed. Reevaluation of the treatment regimen is necessary if a drug is too difficult to tolerate or too complicated to manage[346]. It has become evident that a narrow window exists where drug concentration below a certain level can lead to replication and if the correct mutations occur drug resistance and selection of these variants, while concentrations that are too high can cause toxicities[355]. Despite the fact that HAART is remarkably effective in reducing viral replication, when subjects adhere to their treatment regimen, this therapeutic intervention cannot eradicate the viral infection, as shown by evidence showing the virus rebounds with treatment interruption[358-360].

Due to the prolonged use of these agents, a number of complications arise. Severe metabolic changes occur with prolonged use in a subset of HAART treated patients[361]. Lipodistrophy, or the redistribution of fat deposits, cholesterol and glucose, is a relatively common HAART associated side-effect. Abnormal fat deposits, as well as a deficit of essential fat deposits result from this toxicity[362]. In addition, some antiretrovirals have deleterious effects on mitochondria and thus several tissues are affected by the loss of this energy producing cellular compartment[363]. This form of toxicity can lead to muscle wasting, nerve degeneration, and heart failure. Additionally, this side-effect can have profound effects such as degeneration of the liver and inflammation of the pancreas. Finally, HAART treated subjects appear to have an elevated rate of osteonecrosis, or weakened bones due to the toxic nature of the drugs[363].

Given the difficult nature of the treatment regimens, the potential for resistance, and the HAART associated side-effects, a large dilemma exists in the HIV community as to the optimal time to initiate therapy. As prolonged therapy leads to drug toxicity, a number of groups have speculated that it may be beneficial to postpone initiation of treatment until CD4 counts reach a critical level[254]. On the other hand, it has been shown that initiation of therapy during acute infection may limit the seeding of viral reservoirs in tissues and potentially protect HIV specific CD4+ T helper cells from the cytopathic effects of the virus[318,364]. Treatment in acute infection has resulted in the preservation of proliferative responses[238]. However there are concerns regarding prolonged exposure to HAART due to starting therapy so early in infection given the problems arising from toxicities and side-effects. Thus a great deal of interest is focused around the issue of the ideal time to treat in order to minimize the negative impact of prolonged exposure to

therapy while still achieving the beneficial immune preservative effects of therapy in early treatment.

As mentioned earlier, early hopes for viral eradication now appear to be unrealistic. Close to a decade of HAART therapy has conclusively shown that this strategy will not be effective in complete viral eradication due to latent reservoirs[258,318]. In addition, effective HAART is complicated and requires both dosage monitoring, vigilance over potential drug-drug interactions and routine physician care in order to assess efficacy and potential adverse events[365]. Side-effects are frequent in as many 74% of adults showing one of the myriad of potential problems associated with the 4 classes of antiretrovirals[365]. An extremely high degree of adherence to therapy is critical to reduce potential viral mutations associated with drug resistance[365]. Finally, the cost of long term usage of HAART may be beyond the means of large proportions of HIV infected patients. Thus recommendations for the delay of therapy initiation have been made in order to reduce long term toxicities and potential viral resistance[348]. In addition, new strategies have been developed in an effort to employ HAART in a manner that would allow for the controlled exposure of the autologous virus to the immune system, with the intention of providing the immune system with sufficient antigen to develop novel protective responses over time[347,366].

1.15 Scheduled Treatment Interruption (STI)

The potential utilization of treatment interruptions as a form of immunotherapy was introduced to the medical community with a report of a patient from Berlin, who was able to control viral replication for over 4 years after cycling on and off therapy twice[367]. This patient initiated HAART, including hydroxyurea (an immune modulator), early in

infection. Control of viral replication, in this patient, was associated with increased T cell mediated activity but no neutralizing antibody production.

Structured treatment interruptions (STIs) became a new potential alternative to long-term therapy usage. Rationale for the use of this treatment method was developed for four separate scenarios: HIV PI, chronic infection, chronic infection with immunotherapy, and as a form of salvage treatment.

In the setting of acute infection, as seen in the Berlin patient, interruptions in therapy initiated in early infection, which preserved HIV specific CD4+ T cell activity, could lead to controlled intermittent exposure to autologous virus allowing for broadening of the immune response while the immune system was still relatively intact, potentially leading to immune system boosting[347,366,368]. In the context of chronically infected patients, short monitored interruptions in treatment could lead to potential immune reconstitution, with boosting of both CD4+ and CD8+ HIV specific cell mediated immunity[347,366,368]. The third target population included patients that had failed all their antiretroviral drug regimens due to viral mutation acquired drug resistance. The theoretical approach in this setting was that interruptions in therapy would allow for the emergence of wild-type virus which is presumably more fit than drug resistant variants. This drug sensitive virus would then be susceptible to therapy. As the drug resistant mutant reemerges on therapy, therapeutic interruption would lead once again to the reemergence of the wild type species. Therefore alternating competition between viral species, may allow patients to have less drug exposure, and better control of viral replication and associated pathogenesis[347,368]. Finally, structured treatment interruptions

could provide a cost-effective alternative to patients that are unable to afford long term HAART therapeutics[347,368].

STIs in the setting of PI have proven to have moderate success. A trial conducted on 8 acutely infected patients, resulted in spontaneous control of viral replication in all subjects with one or more cycles of on and off therapy[238]. Additionally, STIs conducted in a group of acutely SIV infected monkeys, was performed to assess if treatment interruption, and not other biological factors, were responsible for spontaneous control of viral replication at the end of STI. Monkeys were separated into an STI arm and a continuous HAART arm. Data from this study revealed conclusively, that the enhanced broad CTL mediated viral control, which only developed in the monkeys in the STI arm of the study, was responsible for control of viral replication in the absence of therapy[369].

STI in the context of chronic infection has not proven to be as successful. While it appears that immune responses can evolve during STI, these responses do not appear to control viral replication[370,371]. Disappointing data emerged from a large study conducted on chronically infected patients in Europe, as minimal improvement in viral control or CD4 T cell numbers were observed in the 133 patients enrolled[372]. Yet, 5 patients undergoing STI in Philadelphia, exhibited immunological reconstitution, as both CD4+ and CD8+ responses emerged following the study, despite a lack of spontaneous viral control[373]. In another study 11 subjects undergoing seven cycles on and off therapy, showed increases in T cell mediated immune responses during viral rebounds, but the immunity was not sufficient in controlling viral loads for extended periods of time[374]. Finally, STIs conducted in 12 patients in Spain effectively increased viral doubling times

during treatment interruption, but were not able to mediate long term control of viremia[375].

Given their dissimilar strengths and weaknesses, the combination of STI and therapeutic vaccination has been proposed for use in the context of both acute and chronic HIV infection[370,376,377]. Therapeutic immunization with Remune, an inactivated HIV recombinant A/G isolate from Zaire missing gp160 proteins, does not appear to induce novel CTL responses, rather this strategy has been demonstrated to be effective in the induction of HIV specific CD4+ proliferative responses in the context of HAART therapy in chronic infection[370]. Furthermore, STIs have been shown to be effective in the induction of greater diversity of novel immune responses in both acute[238] and chronic infection[373], yet another study has demonstrated little benefit to the CTL arm of the immune response and has only observed enhanced proliferative activity in remune immunized subjects[370]. Thus hopes to induce both protective HIV specific CD4+ (remune specific) and CD8+ (autologous) responses may be attainable via the combination of STIs and remune based therapeutic strategies.

STIs, used in the setting of drug failure, have been disappointing. Patients in two separate studies conducted to assess the efficacy of this modality experienced little to no immunological benefit. Despite a reemergence of wild type virus in two thirds of tested subjects, 25% of patients did not recover their pre-interruption CD4 cell numbers, and 75% of patients exhibited viral rebounds following treatment re-initiation[378]. Similarly, a second study conducted on patients on failing regimens, demonstrated that in spite of a reversion to wild type virus in the majority of patients off therapy, resistant virus was still detectable, and resulted in little to no susceptibility on therapy re-initiation[358].

A study conducted at the NIH, to explore the possibility of short interruptions in HAART with sustained viral control and no resistance mutations, enrolled patients for 12 cycles of 1 week on and 1 week off therapy[379]. Interruption and re-initiation intervals were selected based on viral kinetic studies, which established that viral rebound does not take place within the first 7-14 days off therapy. Given this lag in viral replication, the study demonstrated that all patients maintained control of their virus during the cycling, despite the usage of 50% less drug than before. Patients experienced no net loss of CD4+ or CD8+ T cells, and viral loads remained below 50 copies/mL. Given these results concerns with respect to cost, tolerance, and adherence issues may be slightly alleviated.

1.16 Vaccines

Despite the success of HAART in reducing mortality due to AIDS in the developed world, multiple difficult obstacles are impeding the introduction of these medications to the developing world, where the majority of infections occur. Additionally, given that eradication of the virus is unlikely and due to the long term toxicities associated with prolonged treatment new strategies are essential for the treatment of this infection worldwide. Thus it is a long term goal of the HIV community to develop therapeutic and prophylactic vaccines that could stimulate the immune system sufficiently to lead to life long control of the viral infection, as is seen for EBV and CMV. Clinical information on the course of disease and associated immune pathogenesis are necessary for the design of a number of potential vaccines currently under intense investigation. Correlates of immune protection are still unclear and the relevance of the animal models used in vaccine research to human HIV infection are two issues that once resolved will greatly aid in the development of an effective vaccine.

1.16.1 The animal model:

HIV does not infect small laboratory animals. Other than humans, the virus appears to only cause disease in chimpanzees. Ethical issues relating to the use of this endangered species preclude their use in research. An important step in the development an animal model was the discovery that an HIV related virus, SIV, causes a similar disease to HIV in rhesus macaques. The use of these monkeys has contributed greatly to the characterization of the immunopathogenesis associated with HIV infection, unraveling the mysteries behind immune protection, and in the development of vaccine strategies that can boost and protect against virus pathogenesis[380].

Although SIV and HIV are relatively homologous in sequence, a great deal of heterogeneity exists in the envelope gp120 glycoprotein. This divergence has resulted in a number of difficulties in assessing whether vaccines that induce neutralizing antibodies to HIV can be effective in suppressing HIV infection. The development of a chimeric strain of the virus that expresses the HIV envelope protein on an SIV virus, SHIV, has allowed researchers to overcome these problems[380].

Concerns of whether the SIV model represents a true picture of the clinical course of disease in humans has been expressed[380]. Several points of contention between the two systems exist. Firstly, infection of monkeys with laboratory synthesized isolates is not a true representation of the population of clinical isolates that exist in the human population. Laboratory isolates have been developed to induce a uniform disease course, which is not representative of the varying patterns in the human population. Secondly, monkey studies employ concentrations of virus that are 10-100 fold higher than what is transmitted during human infections. Thus the degree of protection seen in monkeys may

not be a true indicator of protection for human infection. Thirdly, as few HLA alleles have been characterized in the monkey population, compared with the wealth of information on human MHC alleles, many studies have been conducted on monkeys expressing a single well characterized Mamu A*01 allele in an effort to study the immune response. Given the high degree of polymorphism that exists in the human population, with respect to the distribution of HLA alleles, focusing on a single allele - which may be associated with protection or susceptibility, may lead to biased data.

1.16.2 Old vaccines:

Altered non-pathogenic live virus as a vaccine has been successful in the control of polio, measles, and chicken pox. The utility of this vaccine strategy for HIV was explored in the early 1990s when an attenuated version of SIV was given to a group of monkeys. Attenuation was induced via a small deletion in the Nef gene. Vaccinated monkeys were protected from challenge with wild type virus[381]. An alarm was sounded when, on further investigation of the monkeys, it became apparent that the virus was able to reconstruct the deleted region of the Nef gene product and became pathogenic in these monkeys[382]. Additionally, given the virulent nature of Nef deleted viral strains in neonatal macaques, vaccine efforts based on live attenuated HIV strategies were discontinued[383].

The inactivated virus strategy that is used to induce immunity and protection for polio and influenza, was attempted in an HIV vaccine in the late 1980s. Inactivated HIV does not appear to elicit effective immune protection, largely due to the fact that killed virus is not able to induce T cell mediated immunity although it can induce isolate specific humoral immune responses[384]. As the inactivated virus is processed via the

exogenous pathway, it became clear that this was potentially the reason that CTL responses were not induced.

Use of recombinant proteins has been equally dissapointing. This vaccine strategy, which is effective in the prevention of HBV infection, appears to only confer protection against homologous strains of the virus[385]. Vaccines consisting of recombinant proteins have focused on delivery of the Env glycoprotein gp120 or gp160, in order to induce neutralizing antibodies. Vaccines, based on this strategy induce proliferative responses, yet are poor at eliciting CTL activity and have had little effect on neutralizing clinical isolates[386,387].

1.16.3 New Vaccines:

Adenoviral vectors are under study as a novel potential platform for the delivery of HIV genes. These vectors have been shown to be highly immunogenic and have been successful in inducing protective responses in the context of other viral infections[388]. Moreover, gene deleted attenuated adenovirus has proven to be immunogenic and has been demonstrated to lead to protective responses in immunized monkeys against SHIV induced disease[389]. There are concerns that the vector itself may be or become an immune target, such that vaccine boosts will be impeded by antibody production or vector induced cell mediated immunity. These concerns may be overcome using high titer doses of the viral vector[389]. Furthermore, while Adeno-associated virus has been able to induce potent SIV specific responses in monkeys, the fact that this virus integrates into the host genome has raised concerns[384].

Two new strategies for vaccine design are underway, the use of live attenuated vectors and DNA plasmid technology. Immunization with recombinant vaccinia has had

great success in the context of inducing both HIV specific cellular and humoral immunity in the non-human primate model[390]. Combination of this strategy with an HIV subunit protein boost, leads to protection against several SIV isolates[391]. Concerns pertaining to potential vaccinia replication in immunosuppressed populations have given impetus to the development of live vectors that are more attenuated than vaccinia. These efforts have focused on canary pox based vectors and a more attenuated modified vaccinia virus Ankara (MVA). MVA based vaccines have been effective in protecting immunized monkeys against SIV or SHIV[392,393]. Low titers of antibodies were detected in 70% of vaccinated monkeys, proliferative responses were detected, and 29% of monkeys had detectable CTL activity. In parallel, efforts are underway in assessing the efficacy of a second multiply deleted vaccinia vector platform, NYVAC[394,395].

Plasmid DNA delivery expresses encoded proteins and is capable of inducing both humoral and cell mediated responses. Several studies have shown the ability of this vaccine modality to induce protective responses to SIV and SHIV[396,397]. Additionally, use of viral DNA delivery has been combined with cytokine genes, such as IL-2 or IL-15, to augment the immune response[393,396].

Combination of DNA and attenuated viral vector vaccines has demonstrated the critical nature of the induction of virus specific CTLs which correlate with viral containment following challenge in immunized monkeys[398,399]. Prime/boost strategies have successfully induced memory responses that can control viral infection occurring via a mucosal route[398]. In an effort to elicit broad immune responses to efficiently control viral replication, this study employed a DNA prime construct containing Gag, Pol, Vif, Vpx and Vpr SIV sequences and a boost containing Gag, Pol and Env. Yet only effector

responses targeting Gag were ascertained. Given that broad immune responses may be more effective in controlling viral replication in the eventuality that an escape from a dominant response occurs, it would be essential to assess if this strategy can effectively induce immune responses targeting other gene products.

Intense efforts directed at developing an effective vaccine, have shown that the majority of immunized animals are not protected from infection. It appears that these vaccines may provide a means to modulate the immune system to allow animals to live for varying periods of time relatively disease free following seroconversion[384,393]. While all control animals exhibited profound CD4+ T cell depletions following challenge with the pathogenic SHIV 89.6[400], cytokine-augmented DNA vaccinated animals developed potent CTL responses and maintained control of viral replication for 140 days following challenge[396]. Long term follow up of the vaccinated monkeys has demonstrated that seven of the 8 monkeys maintained long-lasting control over viral replication, preserving CD4+ T cell numbers over the course of 2 years. Conversely, one monkey that initially appeared to control viremia, eventually lost control of viral replication and experienced a rapid loss in CD4+ T cell counts[401]. Longitudinal analysis of the Gag sequence revealed the preferential incorporation of an escape mutation within the immunodominant p11C peptide targeted by the monkey's Mamu A*01 allele[401].

Given the late escape of the Mamu-A*01 dominant P11C response, further efforts were invested in determining the effect of CTL pressure on vaccine efficacy using an SIVmac239 Gag DNA vaccine in 9 Mamu-A*01 monkeys[402]. While 1 control monkey died soon after viral inoculation, due to rapid disease progression, 4 monkeys all harbored viral strains that had incorporated the escape mutations in two dominant Mamu-

A*01 targeted epitopes, Gag p11C and Env TL9. Of the 4 immunized monkeys, one controlled viral replication to undetectable levels throughout the 3 year follow-up. PCR amplification was not possible due to the exceedingly low viral levels, and thus investigation of epitope sequence was impossible for this monkey. The remaining 3 immunized monkeys all demonstrated the presence of a threonine to serine at position 2 associated with the CTL escape mutation in the Gag p11C epitope, and a proline to threonine at position 3 mutation in the Env TL9 epitope, coincident with viral break through. Thus although the aim of an effective vaccine would be to induce CTL responses that would lead to the spontaneous control of viral replication, the induction of narrow dominant CTLs, which induce viral escape, may be an important limitation in vaccine design efforts.

1.17 Rationale:

Given the complexity of the immune response targeting HIV, it is critical that we gain a better understanding of the evolution of the host antiviral effector response during the course of infection. Based on the strong association between CTL activity and viral control throughout infection, we have generated data on the pattern of the immune response at various stages of infection on and off therapy following infection. Data generated on the fate of the immune response throughout infection will certainly prove to be essential for proper patient management and for effective vaccine design.

Chapter 2: Cross-Sectional Analysis of HIV-Specific Effector Responses in HIV PI

HIV-SPECIFIC EFFECTOR CD8+ T CELL ACTIVITY IN PATIENTS IN PRIMARY HIV INFECTION

Running Head: HIV-specific effector responses in primary HIV infection

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2.1 ABSTRACT

The Interferon-γ Elispot assay was used to assess and compare the magnitude and breadth HIV-specific CD8+ T cell responses in treatment-naïve subjects in the first year of HIV primary infection and the chronic phase of infection. Twenty-five subjects tested within a year of exposure to HIV resulting in seroconversion and 20 subjects in chronic infection were screened for HIV-peptide specific activity by stimulating peripheral blood mononuclear cells (PBMC) with a panel of 5 to 21 (mean 11.2 ± 3.5) HLA class I restricted HIV peptides. A significant correlation was observed between the magnitude and breadth of HIV-specific effector responses and time elapsed from exposure ($r=0.63$ for magnitude versus time and $r=0.64$ for breadth versus time, $p<0.02$; t-test). Maximal breath of the HIV gene product reactivity was achieved within 2 months of exposure for Nef-specific responses and by 4 months of exposure for responses directed to Env, Gag and reverse transcriptase.

2.2 INTRODUCTION

High levels of viral replication followed by induction of an immune response are characteristic features of human immunodeficiency virus (HIV) primary infection (PI) [1,2]. In PI, HIV-specific effector cells have been found as early as a few days after presentation with an acute seroconversion syndrome and weeks before the detection of neutralizing antibodies[3]. HIV-specific CD8+ effector cells appear to play an important role in controlling viral replication during PI. For both acute HIV and simian immunodeficiency virus (SIV) infection, the appearance of HIV- or SIV-specific cytotoxic T lymphocytes (CTL) is temporally associated with a dramatic fall in plasma viremia[3-5]. Furthermore, the absence of HIV-specific CTL3 or the development of a narrow oligoclonal primary immune response repertoire during PI[6] is associated with poor prognosis. The most direct evidence that CD8+ T cells are involved in suppression of viral replication *in vivo* in PI comes from rhesus macaque models. Animals depleted of CD8+ T cells before infection with SHIV chimeric virus develop higher plasma viremia higher levels of virus in lymphoid tissue and more profound immunosuppression than control animals[7]. CD8+ CTL effector cells are also implicated in viral control during the chronic phase of infection where an inverse correlation between viral load and the frequency of HIV-specific effector cells specific for certain HIV epitopes has been observed[8]. The presence of strong and broad HIV-specific cytotoxic T lymphocyte (CTL) responses in long term non progressors with low viral loads[9-11] and declining CTL responses in HIV disease progressors[11,12] also support an important role for HIV-specific effector cells in viral control. CD8+ cell depletion of rhesus macaques during chronic

SIV infection results in a rapid and marked increase in viremia that is suppressed with the re-emergence of SIV-specific CD8+ cells[13,14].

Despite the induction of HIV-specific immune responses during PI, HIV is not cleared and eventually establishes a persistent infection. The inability of HIV-specific CD8+ T cells to rid the body of HIV is not completely understood. Studies in several animal and human models of virus infection demonstrate that early virus-specific immune responses influence the course of disease[6,15-18]. These data emphasize the importance of understanding the characteristics of HIV-specific immunity in HIV PI and how it may influence HIV disease course. HIV-specific CD8+ T cells can control HIV replication either through lysis of HIV-infected cells or by secretion of soluble mediators including the cytokines interferon-gamma (IFN-γ) and tumor necrosis factor α (TNFα), the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and regulated upon activation normal T cell expressed and secreted (RANTES) and the cytotoxin, perforin[19-23].

We report here data characterizing HIV-specific effector cell reactivity in 19 subjects screened within 4 months of exposure to HIV that resulted in establishment of infection. HIV-specific effector reactivity in this group is compared with that in 9 individuals tested later in the first year of HIV infection and 20 chronically HIV infected individuals. We evaluated the breadth and magnitude of HIV-specific CD8+ T cell recognition to panels of major histocompatibility complex (MHC) class I restricted peptides derived from HIV-1 *env*, *gag*, *pol*, and *nef* sequences using an IFN-γ Elispot assay. This method for measuring the frequency of HIV-specific CD8+ T cells appears to correlate well with direct peptide-specific cytolysis and detection of HIV-specific T cells

using HLA class I-peptide tetramer complexes binding defined T cell receptors[23-26]. Furthermore, the ability of individual cells to secrete IFN-γ several hours after stimulation and differentiate into individual CTL clones further supports the functional relevance of this assay for measuring HIV-specific effector activity[27,28].

2.3 METHODS

Study population

We studied 25 male subjects enrolled in the Montreal HIV Primary Infection (PI) Cohort of individuals with documented recent HIV infection. Baseline characteristics of the PI study population are presented in Table 1. No subject had received any antiretroviral therapy before the time they were tested. Eligibility criteria for the PI study included negative tests for antibodies to p24 antigen by ELISA in subjects with p24 antigenemia; evolving humoral immune response with positive results for anti-p24 antibody ELISA tests and indeterminate Western blot and p24 antigenemia with emergent positive HIV serology within 3 months; HIV seroconversion within a 6-month period following negative anti-p24 antibody ELISA testing; and/or typical acute retroviral syndrome within the 3 previous months[29].

The presumed date of exposure resulting in infection was estimated using the date of onset of symptoms of an acute retroviral syndrome minus 15 days, or, for asymptomatic PI, the date of the first indeterminate Western blot minus 30 days. Information obtained from questionnaires addressing the timing of high-risk behavior for HIV transmission was used when available to confirm the presumed date of exposure resulting in seroconversion. Assignment of subjects to study groups was supported by laboratory tests showing a non reactive less-sensitive enzyme immunoassay (EIA) HIV p24 antibody test[30].

Two groups of PI study subjects were constituted based on the time elapsed between the presumed date of exposure and the time at which a sample was collected for testing. The early PI subject group was composed of 19 men naïve to highly active anti-

retroviral therapy (HAART) tested for HIV-specific effector activity within 4 months of the presumed date of exposure (median 50 days; range 24 to 110 days). Samples from 9 HAART naïve male subjects were screened at later times in the first year of infection (median 251 days from exposure, range 173 to 285 days). This late PI group included three individuals in the early PI group who refused treatment with HAART while agreeing to be followed and 6 new subjects identified later in the first year of HIV infection. For comparison we also studied 20 asymptomatic age-matched subjects (19 males and 1 female) in the chronic phase of HIV infection. All of these individuals were HAART naïve and had been diagnosed as HIV seropositive a median of 6.1 years (range 1 to 15 years) at the time tested.

HIV-1 antibody testing was performed at three University hospital sites. Western blot analysis for HIV-1 antibodies and p24 antigen capture assays were performed at the Laboratoire de Santé Publique de Québec. Plasma viremia was measured using the Roche Amplicor assay (Roche Diagnostics, Mississauga, Ontario, Canada) with a limit of detection of 500 HIV-1 RNA copies/ml plasma. Plasma samples falling below this limit of detection were retested using the ultrasensitive method (Ultradirect, Roche) with a limit of detection of less than 50 copies/ml. T cell subset distribution was measured by flow cytometric analysis.

Cells and peptides: PBMC were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsala, Sweden) and frozen in 90% fetal calf serum (GIBCO BRL Life Technologies, Burlington, Ontario, Canada), 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis MO). Subjects were typed for MHC class I antigen expression by the

amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI)[31]. HLA-A2 positive individuals were subtyped using high-resolution primer sets to identify the individuals who were A*0201. Genomic DNA for molecular HLA-typing was prepared from either fresh blood or Epstein-Barr virus transformed B (EBV-B) cell lines using the QIAamp DNA blood kit (Qiagen Inc. Mississauga, Ontario, Canada). The HIV-1 epitopes used for PBMC stimulation were chosen from the National Institutes of Health (NIH) HIV Molecular Immunology Database[32]. Peptides of 9-, 15-, or 20-amino acids in length containing these sequences were obtained from the Medical Research Council AIDS Reagent Project (Hertz UK) and the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Lyophilized peptides were diluted to 1 mg/ml in Hank's buffered saline solution (HBSS); 10% DMSO and stored at –20°C. They were used for stimulation at a final concentration of 20 μM.

Elispot assay for single cell IFN-γ release: IFN-γ secretion by virus-specific CD8+ T cells was quantitated by the Elispot assay. Frozen PBMC were thawed and stimulated in microfuge tubes with 20 μM of peptide for 3 hours before being seeded at 2 x 10^5 and 5 x 10^4 cells per well in duplicate into 96-well polyvinylidene difluoride backed plates (MAIPS 45; Millipore, Bedford MA) coated with 5 ug/ml of anti-IFN-γ mAb (1-DK-1; Mabtech AB, Stockholm, Sweden). Stimulating peptides were chosen based on their having amino acid sequence binding motifs for at least one of the MHC class I alleles expressed by the individuals being tested. Negative controls for these experiments consisted of stimulation with no peptide whereas positive controls consisted of

stimulation with anti-CD3 (Research Diagnostics, Inc., Flanders NJ). Stimulated cells were incubated 16-20 hours at 37°C in 5% CO_2. After washing off the cells, IFN-γ secreting cells were detected as spots by sequential addition followed by washing of a second biotin-conjugated anti-IFN-γ antibody (7-B6-1-biotin, Mabtech) diluted 1:2000, streptavidin alkaline phosphatase (Mabtech) diluted 1:2000 and 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP) p-nitro blue tetrazoliumchloride (NBT) substrate (Bio-Rad Laboratories, Richmond, CA). Spots were counted using a stereomicroscope (Carl Zeiss Canada, New York, Ontario, Canada). The number of spot forming cells (SFC) per million PBMC was calculated based on the number of input cells per well responding to antigen stimulation. Negative control results have been subtracted from the results reported. Negative control stimulation produced less than 5 spots per well in greater than 90% of experiments. In experimental wells the signal was considered positive if at least 10 spots per well over background was present in the wells plated with 2×10^5 cells, the number of spots obtained was proportional to the number of cells plated and the number of spots per well was at least 2-fold greater than the negative control wells. The identity of IFN-γ secreting cells as CD8+ was confirmed by the reduction of SFC numbers generated to all peptides stimulating above background responses following depletion of CD8+ cells with magnetic beads (Dynal, Lake Success, NY).

Statistical analyses: The significance of proportional between group differences in reactivity to all peptides in the screening panel and peptides derived from HIV gene products was tested by χ^2 analyses. The significance of between group differences was tested by the unpaired student t-test. The significance of the correlation coefficient

between the magnitude and breadth of the HIV-specific immune response and time from the presumed date of exposure was tested by the paired t-test. P-values of less than 0.05 were considered significant.

2.4 RESULTS

Table 1 provides information on the age, sex, mode of HIV transmission, MHC class I type and whether symptoms of an acute retroviral syndrome were reported for each subject in the PI study groups. Table 1 also lists the estimated time from exposure resulting in seroconversion, absolute CD4 and CD8 counts and viral load at the time samples were screened for HIV-specific immune responses. The 25 HIV PI study subjects had a median age of 41.6 yrs (range 26 to 53 yrs) and the chronically infected individuals had a median age of 39.3 yrs (range 20 to 55 yrs). The early PI, late PI and chronically HIV infected groups were similar in age, CD4+, and CD8+ cell counts. The three groups were also similar in composition with respect to route of HIV acquisition. The median $\log_{10}$ viral load was 5.45 [range 2.9 – 6.6], 4.8 [range 3,7 to 5.5] and 4 [range 1.7 to 5.5] for the early PI, late PI and chronically HIV infected groups, respectively. The early PI group had a significantly higher viral load than the chronically infected group ($p=0.018$; unpaired t-test). Higher viral load in the early PI compared would be expected as this population contains a larger proportion of individuals with transient high levels of viremia characteristic of early infection[33,34].

HIV-specific IFN-γ secreting cells. HLA-restricted T cell responses were evaluated by the IFN-γ Elispot assay. The sequence of the synthetic peptides corresponding to HIV Env, Gag, RT and Nef used as stimuli are shown in Table 2. Fig. 1 shows for 4 representative subjects in each group the number of SFC generated following stimulation with the HIV peptide panel they were screened with. A median of 11 peptides was tested per individuals (range 5 to 21) depending on the HLA type. The number of HIV peptides

used to stimulate PBMC from individuals assigned to the 3 study groups was similar (unpaired t-test). There were no between group differences in two factors that could have influenced the selection of HIV peptides for testing, i.e. the proportion of individuals having homozygosity at any of the MHC class I loci or the proportion of subjects expressing MHC class I alleles having an allele frequency of less than 5% or 1% in the predominantly Canadian Caucasian population from which the study subjects were drawn[35]. HIV-specific responses to at least one peptide were detected in 94% of subject in early PI, all subjects in late PI and 90% of subjects in the chronic phase of infection.

Early and late PI study subject groups were compared with each other and with the group in the chronic phase of infection for the breadth of their HIV-specific reactivity by testing for significant differences in the proportion of HIV peptides tested in each group that stimulated a positive response in an Elispot assay. The overall breadth of HIV-specific reactivity was greatest for the subjects in late PI, who reacted with 83 of 113 (73%) of the peptides tested. This was a significantly greater proportion than the 145 of 231 (63%) of peptides that stimulated a response in the early PI subjects group ($p=0.05$; χ^2-test) or the 98 of 220 (45%) of HIV peptides tested that stimulated a response in the chronic HIV infection group ($p<0.001$; χ^2-test) (Table 3). The early PI group also reacted with a greater proportion of HIV peptides than did the group in the chronic phase of HIV infection ($p<0.001$; χ^2-test) (Table 3).

The three populations were next compared for reactivity to HIV peptides derived from individual HIV gene products. No between-group differences were detected for the proportion of HIV Env- or Gag-derived peptides able to stimulate a positive response. Fifty-three per cent, 79% and 35% of RT peptides used for screening stimulated

reactivity in the early PI, late PI and chronic HIV infection groups, respectively (Table 3). All between group comparisons for the proportion of RT peptides able to stimulate responses were significantly different (χ^2-test) (Table 3). Although no differences were observed in the proportion of Nef peptides that stimulated responses in early versus late PI subjects, both these groups reacted to a significantly higher proportion of Nef peptides than did subjects in chronic infection (Table 3).

The magnitude of the HIV-specific response for each subject was calculated by adding the frequency of IFN-γ secreting cells per million PBMC reacting to each individual peptide that stimulated a response considered to be above background. The average magnitude of the HIV-specific response for subjects in early PI, late PI and the chronic phase of infection was 2530 ± 2506, 3193 ± 2619 and 1409 ± 1459, respectively. Reactivity per peptide was determined by dividing the total number of HIV-specific IFN-γ secreting cells by the number of peptides able to stimulate above background levels of reactivity. The average reactivity per peptide for subjects in early PI, late PI and the chronic phase of infection was 297 ± 245, 317 ± 186 and 275 ± 162, respectively. Between-group comparisons revealed no statistically significant between group differences for either of these parameters (unpaired t-test).

Although the average magnitude of HIV-specific reactivity in the early PI group did not differ from that in the late PI group, a statistically significant correlation was observed between the magnitude of HIV-specific reactivity and the time elapsed from exposure for the 19 subjects in early PI ($r=0.64$, $p<0.02$; paired t-test [Fig. 2A]). So too, a statistically significant correlation was detected between the breadths of the HIV-specific response, as measured by the per cent of peptides stimulating a response in each

individual, and the time elapsed from exposure for subjects in early PI ($r=0.63$. $p<0.02$; paired t-test [Figure 2B]).

Because these results suggested that the breadth and magnitude of HIV-specific reactivity increased with time in the first 4 months following exposure resulting in seroconversion we reanalyzed the HIV effector data by separating the early PI subjects into 2 subgroups. Breadth of HIV-specific responses were compared between 11 subjects tested within 2 months of the presumed date of exposure and 8 subjects tested between 2 and 4 months of exposure. The breadth of reactivity to the HIV peptide panel tested in the 11 subjects screened within 2 months of infection was significantly lower than that in individuals tested between 2 and 4 months of infection (69 of 134 [51%] versus 76 of 97 [80%] of peptides tested stimulated positive responses, $p<0.001$, χ^2-test) (Fig. 3A). The subgroup tested between 2 and 4 months of exposure also responded to a significantly higher proportion of Env-, Gag- and RT- derived peptides than did the subgroup tested within 2 month of exposure (Fig. 3B-D). In contrast, similar proportions of Nef peptides stimulated responses in both these subgroups (Fig. 3E). By 4 months from the presumed date of exposure, the breadth of the response was maximal for all the HIV gene products tested and was maintained through the first year of infection (Fig. 3A-E)

Eight individuals in the early PI group, 3 separate individuals in the late PI group and 13 subjects in the chronic infection group were HLA-A*0201 positive (Fig. 4). All were screened for reactivity to HLA-A*0201 restricted HIV peptides Gag 77-85 (SLYNTVATL) and RT 476-484 (ILKEPVGVY). Five of the 8 (62.5%) individuals in early PI responded to Gag 77-85 and RT 476-484. No significant proportional between group differences were detected for reactivity to either peptide. When the early and late

PI groups were combined and compared to the chronically infected group for reactivity to these two HLA-A*0201 restricted HIV epitopes, no proportional between group differences were detected for reactivity to Gag 77-85; a non statistically significant trend towards more frequent recognition of RT 476-484 was observed in subjects in the first year of infection versus those in chronic phase of HIV infection (7 of 11 [63%] PI subjects versus 4 of 13 [31%] chronic infection subjects responded to RT 476-484, $p=0.11$; χ^2-test).

2.5 DISCUSSION

We have screened HAART naïve HIV-infected subjects in early and late PI and in the chronic phase of infection for HIV-specific effector responses to a panel of HIV peptides. Our results show that newly infected subjects tested within 4 months of their presumed date of exposure recognized 145 of 231 (63%) of the MHC class I restricted HIV peptides used for screening. This level of responsiveness was significantly higher in those tested between 4 months and 1 year of exposure, as subjects in late PI responded to 83 of 113 (73%) of the HIV peptides tested. When these results were separated according to the HIV gene products the peptides were derived from, the increase in reactivity between the early and late PI study groups was only significant for RT derived peptides. Both PI study groups responded to more HIV peptides than did subjects in the chronic phase of infection. By dividing the early PI study group into subsets of individuals tested within 2 months of exposure and between 2 and 4 months of exposure it was possible to demonstrate that HIV-specific response to Nef-derived peptides are the first to achieve maximal breadth. Maximal breadth of the responses to Env-, Gag- and RT-derived peptides are achieved by 4 months from exposure and they, together with responses to Nef peptides, are maintained in the first year of infection. Although the average magnitude of the overall response and the magnitude of the response per peptide did not differ between groups, a correlation was observed for the early PI group between time elapsed from exposure and both the magnitude and breadth of the HIV-specific response. Several HLA-A*0201 positive subjects in early PI responded to the Gag 77-85 and the RT 476-484 epitopes restricted by this MHC class I allele.

Weak anti- HIV-specific effector activity in early PI has been reported others[36,37]. Dalod et al. used a similar strategy to the one reported here for dating the presumed date of exposure. Their study population had a median time from exposure of 45 days (range 24 to 97 days), which is comparable to the median time from exposure of 50 days (range 24 to 110) for the early PI group described in this report. Eleven of the early PI subjects were tested within 60 days (median 39 [range 24 to 52 days]) of the presumed date of exposure and therefore are at an even earlier stage of infection than the population described by Dalod et al.[36]. Altfield et al. studied a PI population that was largely in the acute phase of infection with negative HIV p24 EIA tests and HIV p24 antigen positivity and therefore at an even earlier stage of infection than most of the individuals studied here. Altfield et al. also reported a weak HIV-specific response in these subjects[37]. Although the PI study population described here had a greater breadth and magnitude of HIV reactivity than that reported by others, the change in the pattern of reactivity to HIV peptides consistent with that published elsewhere[36,37]. Subjects tested within 2 month of exposure reacted to fewer HIV peptides compared to subjects at later times in PI. Weak anti- HIV-specific effector activity observed in early PI may be akin to that reported in early Hepatitis C infection where antigen-specific cells temporarily failed to secrete IFN-γ during the acute phase of infection characterized by high viremia[15]. As viremia resolves this "stunned" antigen-specific cell phenotype disappears.

The comparison group of subject in chronic HIV infection did not exhibit as broad a response as did PI subjects at all but the earliest time from exposure. This reduction in breadth differs from the results reported by Dalod et al. but may be due to differences in the composition of the 2 chronically HIV infected populations with respect

to how long they had been seropositive[36]. One factor that may account for reduced breadth and magnitude of T cell responses in the more advanced chronically infected subjects may be the selection over time of mutations in the infecting HIV viral isolate sequence. Immune responses to the infecting isolate may no longer recognize the peptides used to stimulate responses in the Elispot assay, which have sequences based largely on the HIV HXB2 isolate.

One explanation for the weak HIV-specific immune responses detected in early PI may be the choice of peptides that were used to screen for HIV-specific responses. The Los Alamos molecular immunology database from which the panel of HIV peptides used to screen for HIV-specific immune responses was drawn lists epitopes that are frequently recognized by HIV-infected individuals in the chronic phase of infection. It is possible that epitopes that are dominant in chronic infection are not dominant in PI and vice versa. Using overlapping peptide sets corresponding to entire HIV gene products to screen individuals in acute infection, Altfeld et al. identified several new epitopes not previously recognized in individuals in chronic infection[37]. Goulder et al. reported that the HLA-A*0201 restricted Gag 77-85 SLYNTVATL epitope was not recognized in any of 11 individuals in early PI even though reactivity to this epitope could be detected later in infection[38]. Changes in the dominance patterns of viral epitope recognition with disease stage occur in EBV infection, in which lytic, and not latent, antigen-specific responses dominate during the acute phase of infection[39,40]. There is evidence that a change in the dominance pattern of viral epitope recognition may also occur in SIV infection of macaques[41], and in Hepatitis C infection[15,42].

Twenty-four of 29 (84%) of the Nef peptides used to stimulate PBMC from subjects within 2 months of exposure elicited a positive response. Nineteen of 20 (95%) and 17 of 20 (85%) Nef peptides tested elicited a response from subjects tested between 2 and 4 months of infection and later in the first year of infection, respectively. This is in contrast to the proportion of Nef peptides recognized by subjects in chronic HIV infection in whom only 28 of 54 (52%) Nef peptides tested were recognized. These results suggest that by 2 months, maximal breadth of HIV Nef-specific reactivity had been achieved. CTL recognizing Nef should be able to lyse cells before mature virions are produced and may do so before a sufficient amount of Nef is available to downregulate the expression of MHC class I alleles, which would compromise CTL recognition of other HIV gene products[43,44]. The proportion of reactive peptides derived from HIV Env, Gag and RT increased in subjects tested within the first 2 months of infection compared with those screened between 2 and 4 months of infection. The proportion of peptides from these 3 HIV gene products recognized by individuals in late PI did not increase significantly.

Goulder at al. reported that reactivity to the HLA-A*0201-restricted epitope Gag 77-85 was not observed in a group of 11 HLA-A2 positive individuals tested very early in infection. All had viral loads >750,000; six were HIV seronegative and p24 positive, 2 were ELISA positive and Western blot indeterminate and 3 were seropositive but within 129 days of HIV infection[38]. In one subject, for whom longitudinal samples were available and who did not recognize this peptide at the first time point tested, anti-Gag 77-85-specific reactivity developed later in the second year of infection[38]. These results were interpreted to support the notion that reactivity to Gag 77-85 is not dominant in PI.

In contrast, we found 5 of 8 HLA-A*0201 positive individuals in the early PI group and 2 of 3 HLA-A*0201 subjects in the late PI group who responded to this epitope. Although the two A*0201 positive individuals tested at earliest times from exposure (24 and 28 days) did not respond, a subject tested at 52 days from exposure did respond. Of the 13 HLA-A*0201 positive subjects in the chronic phase of infection 9 (69%) responded to Gag 77-85. This proportion of epitope-reactive HIV subjects is in agreement with data reported by others[8,45-48]. These disparate results may be due to differences in the methods used to detect HIV-specific cells. Goulder et al used intracellular IFN-γ staining and peptide-MHC tetramer assays to detect antigen specific cells. These methods may not be as sensitive as the Elispot assay used here, which can detect as few as 50 to 100 IFN-γ secreting cells/million PBMC. The number of SFC produced following stimulation of the 5 individuals identified as having positive responses to Gag 77-85 was (221, 75, 212, 568 and 282 per million PBMC). This number of IFN-γ secreting cells is likely below the level of detection of intracellular IFN-γ staining and peptide-MHC tetramer assays.

A second HLA-A*0201 restricted epitope was used to stimulate PBMC from HLA-A*0201 positive subjects. RT 476-484 has been reported to stimulate a response in approximately 20% of HIV-infected HLA-A*0201 positive subjects. Four of 13 (31%) HLA-A*0201 subjects in the chronic phase of HIV infection responded to this epitope while 5 of 8 (62.5%) and 2 of 3 (66.6%) HLA-A*0201 positive subjects in early and late PI responded to this epitope, respectively. When both PI groups were combined and compared a non-significant trend was observed towards preferential recognition of this epitope by subjects in PI versus those in the chronic phase of infection.

In conclusion, HIV-specific effector responses are lower in magnitude and breadth in the earliest phase of PI. They achieve their maximal breadth and magnitude by 4 months from exposure. Responses to the Nef are among the first to appear. Our results do not show an absence of reactivity to the HLA-A*0201 restricted Gag 77-85 restricted epitope in subjects in early PI. The issue of what HIV-epitopes are immunodominant in early infection is an important one for vaccine design where the goal is to induce immunity that will play a role in controlling virus. Further study of the specificity of HIV-specific immunity in individuals in early PI is needed, as there is the possibility that these responses will be superior to those occurring in the chronic phase of infection for controlling virus replication. This information will make an important contribution to the design of effecting vaccines for HIV.

Figure 1

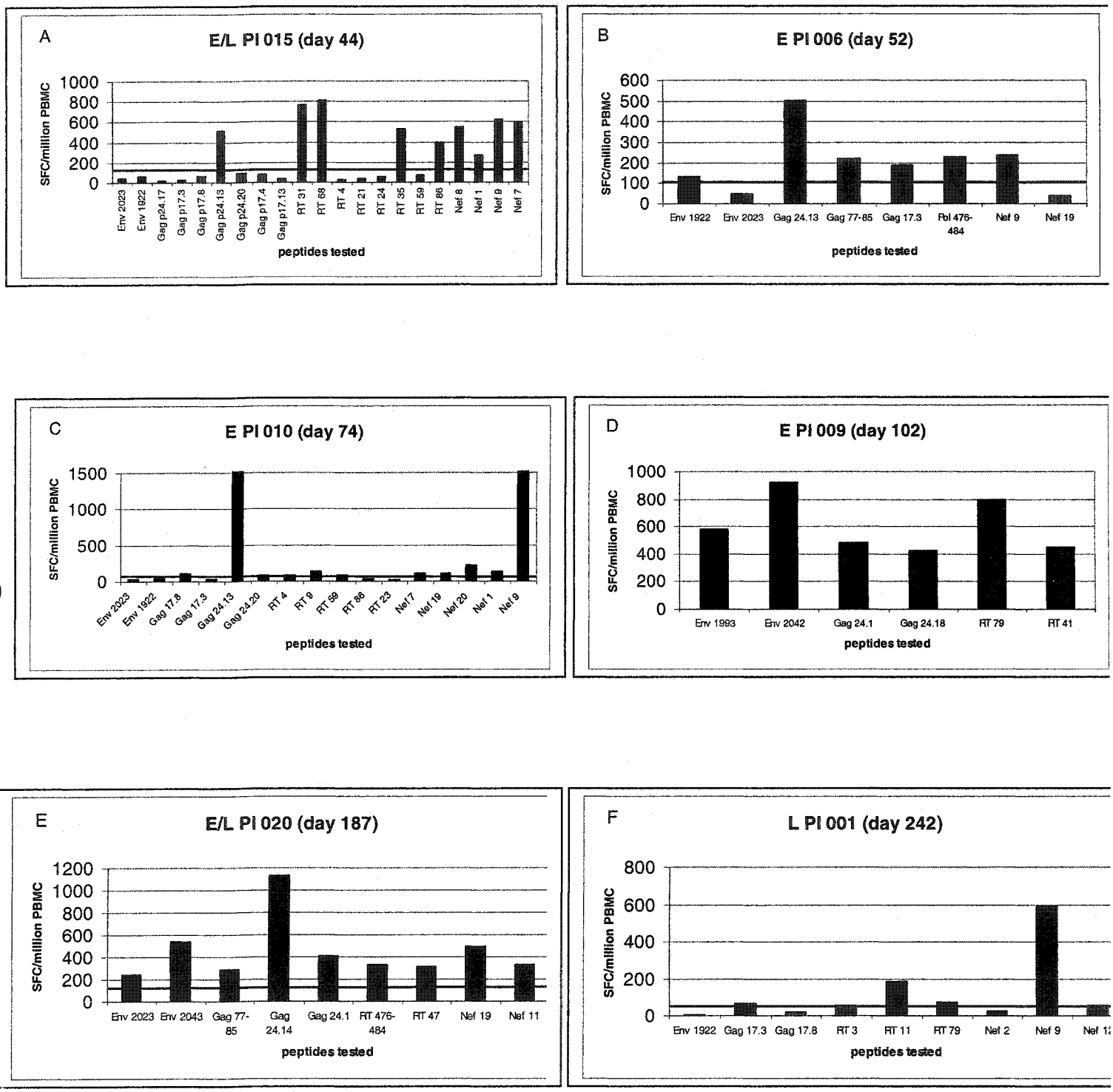
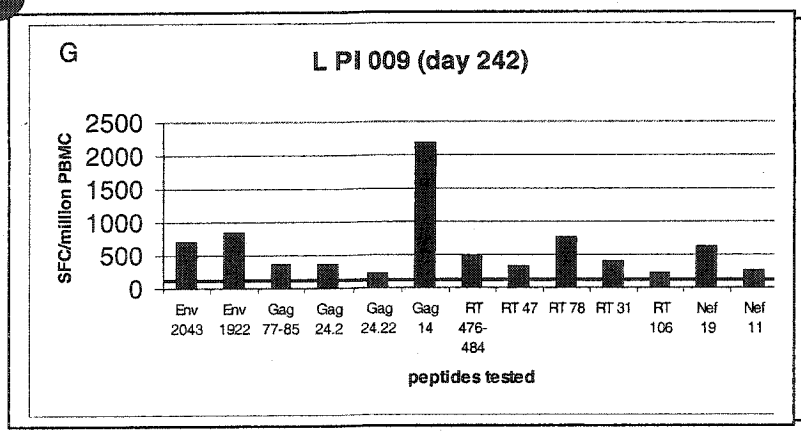
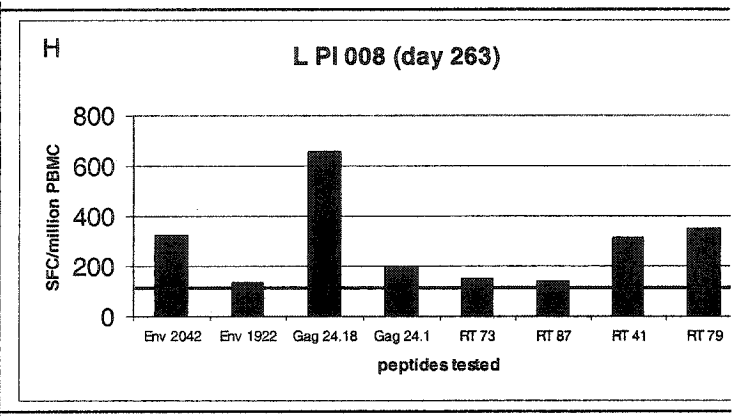
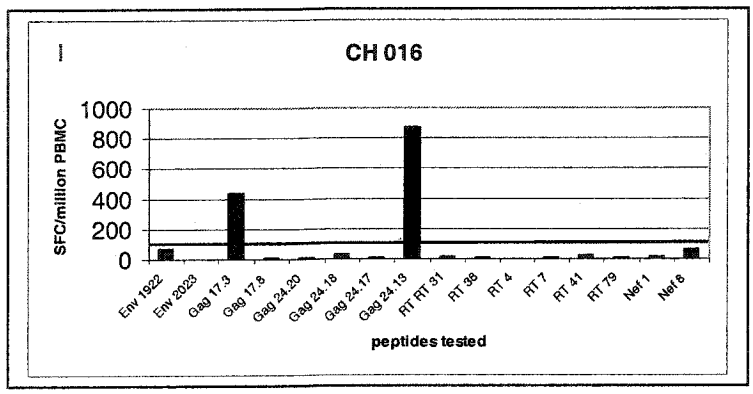
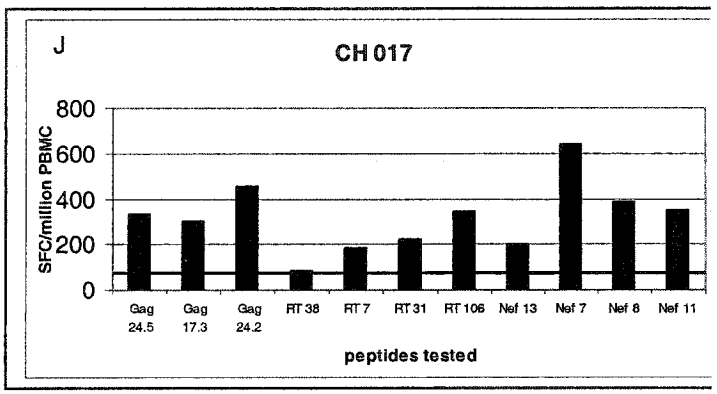

A
E/L PI 015 (day 44)
SFC/million PBMC
1000
800
600
400
200
0
Env 2023
Env 1922
Gag p24.17
Gag p17.3
Gag p17.8
Gag p24.13
Gag p24.20
Gag p17.4
Gag p17.13
RT 31
RT 68
RT 4
RT 21
RT 24
RT 35
RT 59
RT 86
Nef 8
Nef 1
Nef 9
Nef 7
peptides tested
B
E PI 006 (day 52)
SFC/million PBMC
600
500
400
300
200
100
0
Env 1922
Env 2023
Gag 24.13
Gag 77-85
Gag 17.3
Pol 476-484
Nef 9
Nef 19
peptides tested
C
E PI 010 (day 74)
SFC/million PBMC
1500
1000
500
0
Env 2023
Env 1922
Gag 17.8
Gag 17.3
Gag 24.13
Gag 24.20
RT 4
RT 9
RT 59
RT 86
RT 23
Nef 7
Nef 19
Nef 20
Nef 1
Nef 9
peptides tested
D
E PI 009 (day 102)
SFC/million PBMC
1000
800
600
400
200
0
Env 1993
Env 2042
Gag 24.1
Gag 24.18
RT 79
RT 41
peptides tested
E
E/L PI 020 (day 187)
SFC/million PBMC
1200
1000
800
600
400
200
0
Env 2023
Env 2043
Gag 77-85
Gag 24.14
Gag 24.1
RT 476-484
RT 47
Nef 19
Nef 11
peptides tested
F
L PI 001 (day 242)
SFC/million PBMC
800
600
400
200
0
Env 1922
Gag 17.3
Gag 17.8
RT 3
RT 11
RT 79
Nef 2
Nef 9
peptides tested

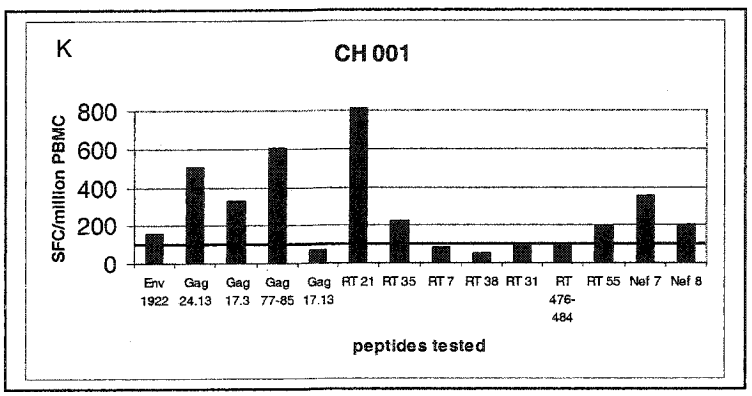
Figure 1 continued











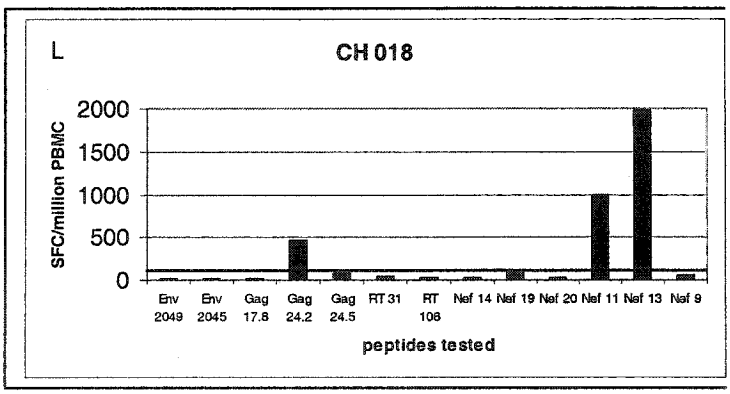
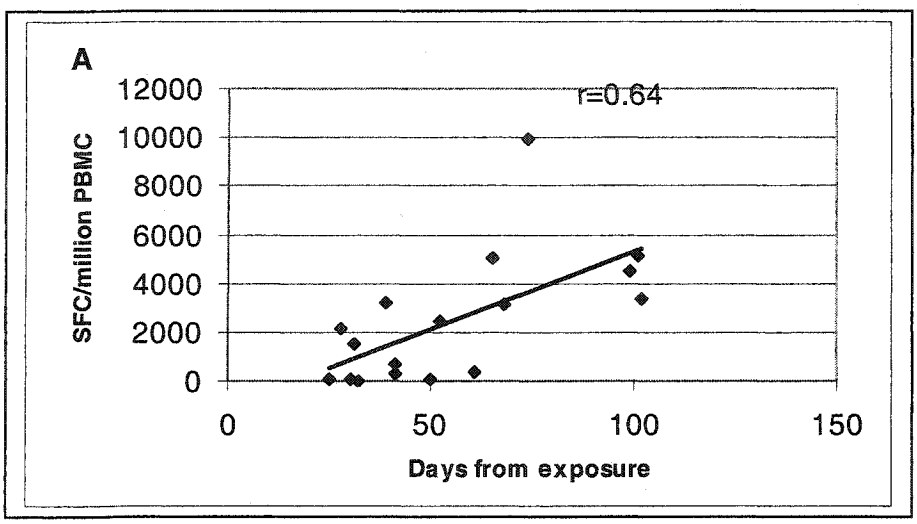


Figure 2


A
12000
10000
8000
6000
4000
2000
0
SFC/million PBMC
r=0.64
0
50
100
150
Days from exposure

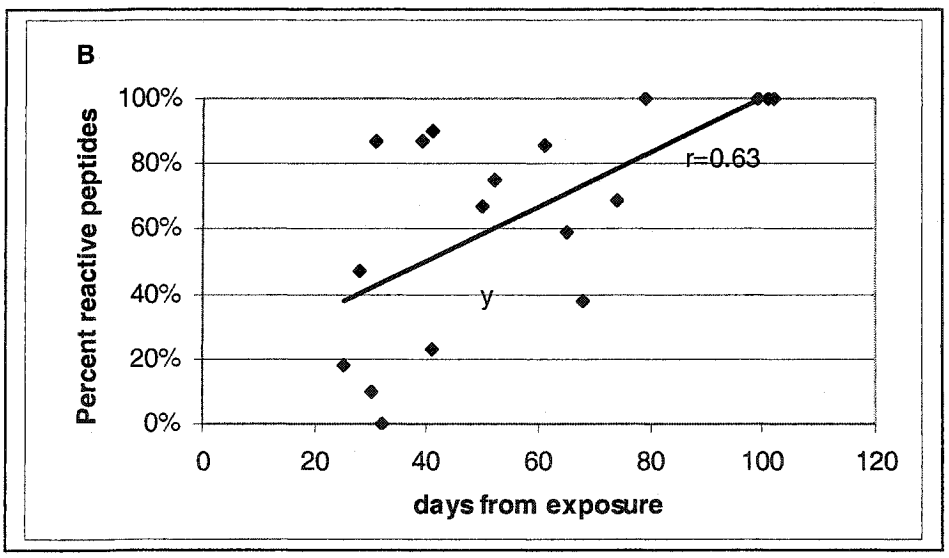
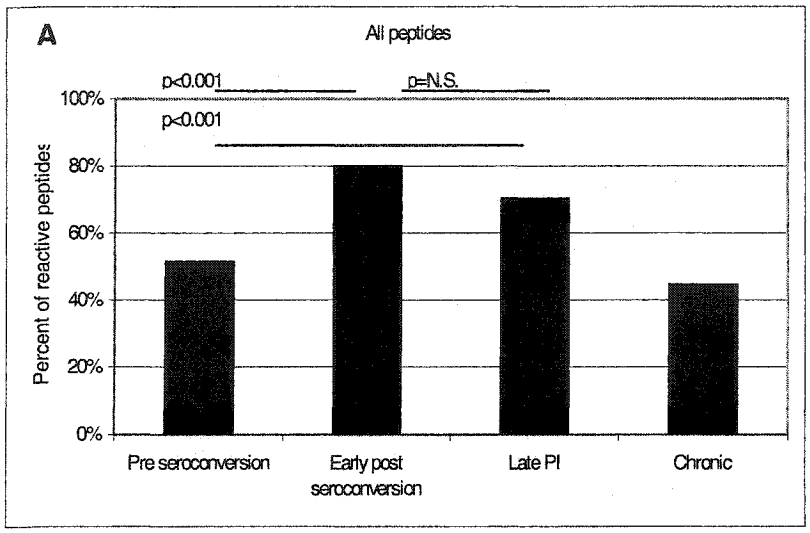
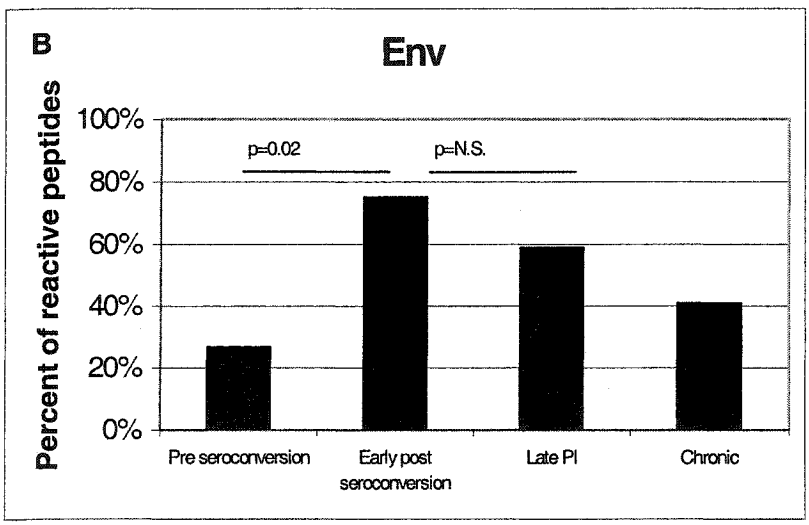

B
100%
80%
60%
40%
20%
0%
Percent reactive peptides
r=0.63
y
0
20
40
60
80
100
120
days from exposure

Figure 2


A
All peptides
p<0.001
p=N.S.
p<0.001
100%
80%
60%
40%
20%
0%
Percent of reactive peptides
Pre seroconversion
Early post seroconversion
Late PI
Chronic


B
Env
Percent of reactive peptides
100%
80%
60%
40%
20%
0%
p=0.02
p=N.S.
Pre seroconversion
Early post seroconversion
Late PI
Chronic

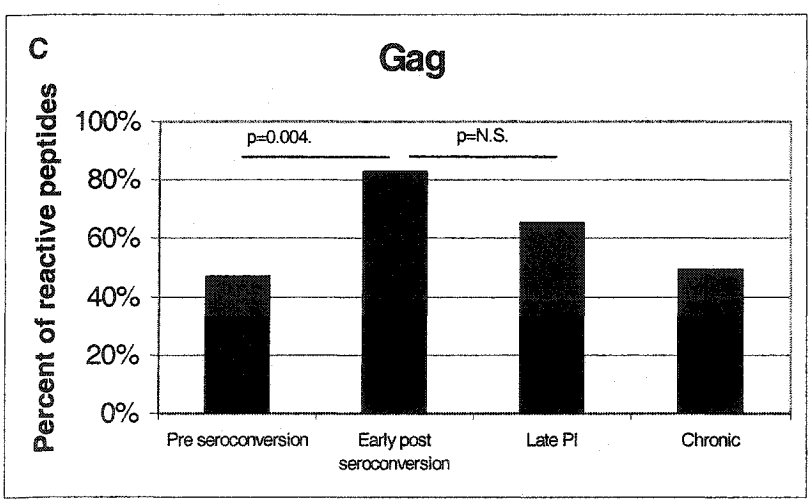
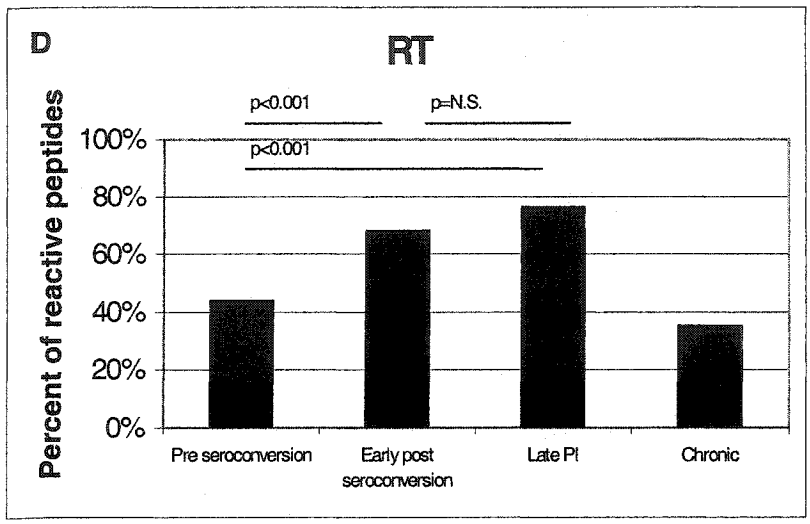

C
Gag
Percent of reactive peptides
100%
80%
60%
40%
20%
0%
p=0.004.
p=N.S.
Pre seroconversion
Early post seroconversion
Late PI
Chronic

Figure 3 continued



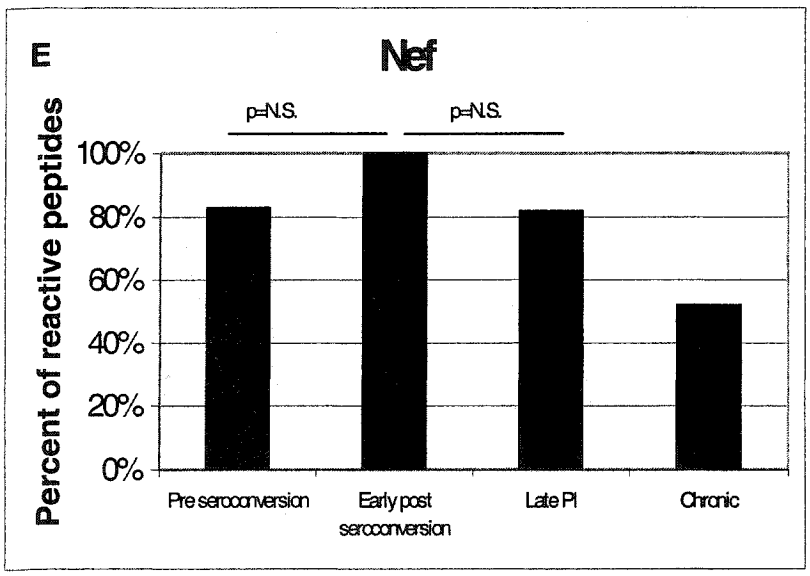
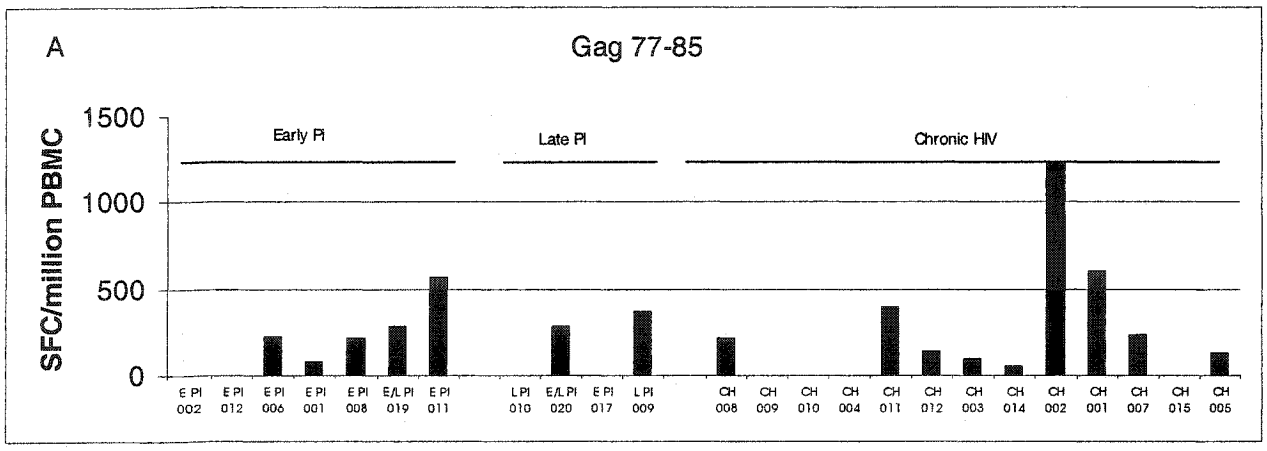


Figure 4


A
Gag 77-85
SFC/million PBMC
1500
1000
500
0
Early PI
Late PI
Chronic HIV
E PI 002
E PI 012
E PI 006
E PI 001
E PI 008
E/L PI 019
E PI 011
L PI 010
E/L PI 020
E PI 017
L PI 009
CH 008
CH 009
CH 010
CH 004
CH 011
CH 012
CH 003
CH 014
CH 002
CH 001
CH 007
CH 015
CH 005

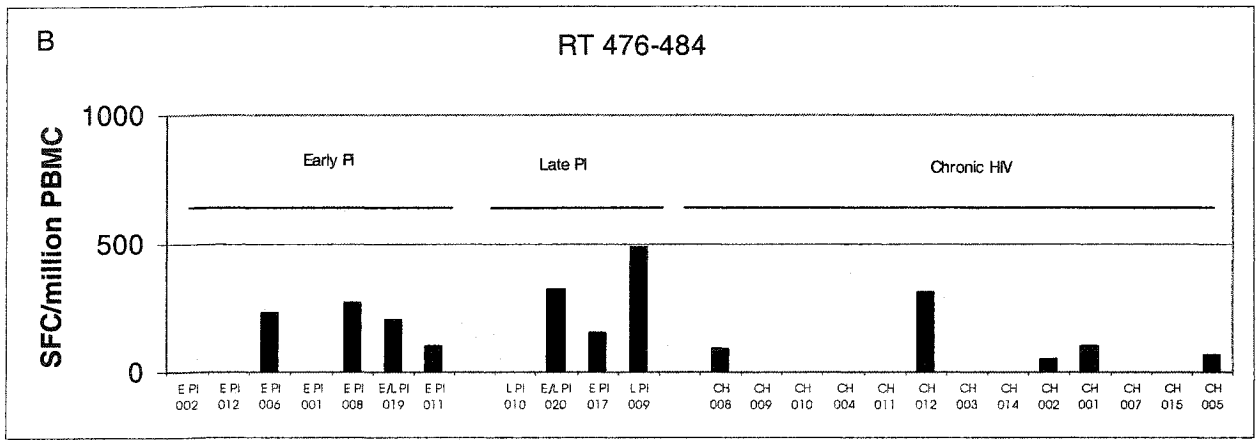

B
RT 476-484
SFC/million PBMC
1000
500
0
Early PI
Late PI
Chronic HIV
E PI 002
E PI 012
E PI 006
E PI 001
E PI 008
E/L PI 019
E PI 011
L PI 010
E/L PI 020
E PI 017
L PI 009
CH 008
CH 009
CH 010
CH 004
CH 011
CH 012
CH 003
CH 014
CH 002
CH 001
CH 007
CH 015
CH 005

Table 1. HIV Primary Infection Study Population Baseline Characteristics

ID	Age/sex	Risk	Symptoms	Estimated time from infection	T cell counts		Log plasma HIV RNA	***HLA type***
					CD4	CD8		
E PI 002	44/M	MSM	Yes	25	612	720	6.0	A2, -, B62, B44, Cw3, Cw7
E PI 012	38/M	MSM	Yes	28	180	1540	6.18	A2, A3, B7, B35, Cw4, Cw7
E PI 013	36/M	MSM	Yes	30	729	1107	4.68	A11, A31, B44, B45, Cw4, Cw6
E PI 003	42/M	HSM	Yes	31	827	2220	5.9	A1, A29, B44, B57, Cw6, Cw16
E PI 010	45/M	MSM	Yes	32	357	4029	6.3	A1, A24, B8, B18, Cw5, Cw7
E PI 004	27/M	MSM	Yes	39	827	2220	5.9	A1, A30, B8, B18, Cw5, Cw7
E PI 005	40/M	MSM	Yes	41	300	447	5.7	A11, A24, B14, B60. -, -
E/L PI 014	29/M	IVDU	Yes	41	430	770	3.8	A3, A66, B18, B41, Cw7, Cw17
E/L PI 015	32/M	MSM	Yes	45	368	1040	4.26	A11, A25, B8, B35, Cw4, Cw7
E/L PI 016	48/M	MSM	Yes	50	580	920	6.23	A11, A23, B7, B35, Cw4, Cw7
E PI 006	53/M	MSM	Yes	52	560	7810	6.65	A1, A2, B8, -, Cw7. –
E PI 001	42/M	IVDU	Yes	61	1140	1090	5.0	A2, -, B18, B44, Cw5, Cw12
E PI 017	23/M	MSM	No	65	432	736	1.8	A2,-,B8, B57, Cw6, Cw7
E PI 018	43/M	Hetero	No	68	467	653	5.45	A3, A23, B35, B44, Cw4
E PI 007	37/M	MSM	Yes	74	228	528	5.08	A1, A30, B8, B51, Cw7, Cw16
E PI 011	41/M	MSM	No	79	350	1458	3.4	A2, A66, B14, B54, Cw1, Cw8
E PI 008	36/M	MSM	Yes	85	956	464	2.93	A2, A68, B39, B57, Cw7, Cw18
E PI 009	43/M	MSM	No	102	840	640	3.2	A23, A31, B44, -, Cw4, Cw5
E/L PI 019	40/M	IVDU	Yes	101	990	810	5.31	A2, A3, B60, B49, Cw3, Cw7
E/L PI 020	39/M	IVDU	Yes	173	500	681	3.7	A2, A24, B27, B40, Cw1, Cw3
L PI 002	41/M	MSM	No	201	309	1237	5.27	A1, A29, B8, B44, Cw7, Cw16
L PI 001	47/M	MSM	Yes	242	440	1110	5.4	A1, A23, B8, B44, Cw4, Cw7
L PI 008	50/M	IVDU	No	254	180	325	5.5	A31, A68, B40, B44, Cw2, Cw3
L PI 009	51/M	MSM	No	263	720	640	4.1	A2, A32, B7, B27, Cw2, Cw7
L PI 010	40/M	MSM	Yes	285	657	542	3.4	A2, A3, B14, B52, Cw8, Cw12

Risk category – MSM = Homosexual exposure; IVDU = exposure through intravenous drug use; hetero = Heterosexual exposure.

Table 2. List of HIV peptides used to screen for HIV-specific effector responses

Peptide Designation	*Location*	*Sequence*	*MHC class I restriction specificity*
Nef-1*	(1-20)	GGKWSKSSVVGWPTVRERMR	B8
Nef-7	(61-80)	EEEEVGFPVTPQVPLRPMTY	A1, B7, B35
Nef-8	(71-90)	PQVPLRPMTYKAAVDLSHFL	A3 , A11
Nef-9	(81-100)	KAAVDLSHFLKEKGGLEGLI	A11, B8, B40, Cw8
Nef-10	(91-110)	KEKGGLEGLIHSQRRQDILD	A1, B60
Nef-11	(101-120)	HSQRRQDILDEWIYHTQGYF	B7, B27
Nef-12	(111-130)	LWIYHTQGYFPDWQNYTPGP	A1, B57
Nef-13	(121-140)	PDWQNYTPGPGVRYPLTFGW	B7
Nef-14	(131-150)	GVRYPLTFGWCYKLVPVEPD	A1, B18, B49
Nef-18	(171-190)	GMDDPEREVEWRFDSRLAF	A25, A66
Nef-19	(181-200)	EWRFDSRLAFHHVAREL	A1, A2, A3, A25, B52
Nef-20	(191-210)	HHVARELHPEYFKNC	A1
RT- 4	(18-26)	DGPKVKQWPLTEEKI	B8
RT- 7	(32-47)	KALVEICTEMEKEGKI	A3
RT- 9	(42-50)	EKEGKISKIGPENPYN	B51
RT- 21	(103-117)	KKSVTVLDVGDAYGS	B35
RT- 23	(113-127)	DAYFSVPLDEDFRKY	B51
RT- 24	(118-132)	VPLDEDFRKYTAFTI	B35
RT- 30	(148-162)	VLPQGWKGSPAIFQS	B51
RT- 31	(153-167)	WKGSPAIFQSSMTKI	A3, A11, B7, B35
RT- 35	(173-187)	KQNPDIVIYQYMDDL	B35
RT- 36	(178-192)	IVIYQYMDDLYVGSD	A2
RT- 38	(188-202)	YVGSDLEIGQHRT-KI	A3
RT- 41	(203-217)	EELRQHLLRWGLTTP	B44
RT- 47	(233-247)	ELHPDKWTVQPIVLP	A2
RT- 49	(243-257)	PIVLPEKDSWTVNDI	B57
RT- 59	(293-307)	IPLTEEAELELAENY\R	B35
RT- 68	(338-352)	TYQIYQEPFKNLKTG	A11
RT- 73	(363-377)	NDVKQLTEAVQKITT	A68
RT- 75	(372-387)	VQKITTESIVIWGKTP	B57
RT- 78	(388-402)	KFKLPIQKETWETWW	A32
RT- 79	(393-407)	IQKETWETWWTEYWQ	B44
RT- 86	(427-442)	YQLEKEPIVGAETFYV	B35
RT- 87	(433-447)	PIVGAETFYVDGAAN	A68
RT- 88	(438-452)	ETFYVDGAANRETKL	A66
RT- 89	(443-457)	DGAANRETKLGKAGY	A29
RT- 90	(448-462)	RETKLGKAGYVTNKG	B14
RT- 106	(528-542)	KEKVYLAWVPAHKGI	B7
RT (476-484)	(476-484)	ILKEPVHGV	A2
p17.3	(21-35)	LRPGGKKKYKLKHIV	A3, A24, B8
p17.4	(31-45)	LKHIVWASRELERFA	B35
p17.8	(71-85)	GSEELRSLYNTVATL	A1, B8
p17.9	(81-95)	TVATLYCVHQRIDVK	A11
p17.13	(121-137)	AAGTGNSSQVSQNY	B35
p17(77-85)	(77-85)	SLYNTVATL	A2
p24.1	(1-20)	PIVQNLQGQMVHQAISPRT-L	Cw3
p24.2	(11-30)	VHQAISPRT-LNAWVKVVEEK	A2, A25, B7, B57

p24.3	(21-40)	NAWVKVVEEKAFSPEVIPMF	B57
p24.4	(31-50)	AFSPEVIPMFSALSEGATPQ	A66, Cw1
p24.5	(41-60)	SALSEGATPQDLNTMLNTVG	B7, B40, B60
p24.7	(61-80)	GHQAAMQMLKETINEEAAEW	B39, B52
p24.8	(71-90)	ETINEEAAEWDRVHPVHAGP	A25, A66
p24.11	(101-120)	GSDIAGTTSTLQEQIGWMTN	B57
p24.13	(121-140)	NPPIPVGEIYKRWIILGLNK	B8, B35
p24.14	(131-150)	KRWIILGLNKIVRMYSPTSI	B27, B62
p24.15	(141-160)	IVRMYSPTSILDIRQGPKEP	B52
p24.17	(161-180)	FRDYVDRFYKTLRAEQASQD	A24, A66, B14, B18, B44
p24.18	(171-190)	TLRAEQASQDVKNWMTETLL	B44, B57
p24.20	(191-210)	VQNANPDCKTILKALGPAAT	B8
p24.22	(211-230)	LEEMMTACQGVGGPGHKARV	A11
Env 1922	(586-598)	EKLWVTVYYGVPVWKEATTT	A3, A11, Cw7
Env 2023	(656-673)	ERYLKDQQLLGF	A24, B8, B14
Env 2043	(786-795)	IVELLGRRGWEVLKYWWNLL	B27
Env 2049	(843-851)	LHIPTRIRQGLERALL	B7

** Peptide designations, locations and sequences refer to those provided by the NIH AIDS Reagent bank and the Medical Research Council AIDS Reagent Project.*

Table 3. Comparison of the ex vivo functional repertoire of antiviral CD8+ T lymphocytes in early PI, late PI and chronic HIV infection

Subjects	Frequency of subjects responding to HIV peptides #responses/#peptides tested (%)					% of responsive subjects [1]	Total SFC number[2]	Mean SFC/peptide[2]
	Env	Gag	RT	Nef	All			
Early PI N=19	11/25[3] (44)	51/83 (51)	41/74[4,5] (55)	43/49[5] (88)	145/231[4,5] (63)	94%	2530 ± 2507	297 ± 245
Late PI N=9	11/17 (65)	24/37[4] (65)	31/39[6] (79)	17/20[6] (85)	83/113[6] (73)	100%	3193 ± 2619	317 ± 186
Chronic infection N=20	9/22 (41)	35/71 (49)	26/74 (35)	28/54 (52)	98/220 (45)	90%	1410 ± 1459	275 ± 162

[1]Per cent of subjects per group with at least 1 HIV peptide-specific reactivity.
[2]Mean ± standard deviation.
[3]Number of reactive peptides/number of peptides tested (percent reactive peptides).
[4] $p<0.05$ Early versus late PI.
[5] $p<0.05$ Early versus chronic HIV infection.
[6] $p<0.05$ Late versus chronic HIV infection.
SFC = spot forming cells; PI = primary infection.

Figure 1. Magnitude of responses to individual human immunodeficiency virus (HIV) peptides used to screen for HIV-specific CD8+ effector cells. Peripheral blood mononuclear cells (PBMC) from subjects in early primary infection (PI) (panels A-D), late PI (panels E-H) and chronic phase of infection (panels I-L) were stimulated individually with the HIV peptides listed on the x-axis. The height of each bar indicates the number of spot forming cells (SFC) that were generated in response to each stimulus.

Figure 2. Correlation between HIV-specific effector activity and time elapsed from exposure to HIV resulting in seroconversion. Nineteen subjects were screened for HIV-specific effector responses by Interferon-γ (IFN-γ) Elispot assay within 4 months of their presumed date of exposure. Panel A displays the magnitude of the HIV-specific response versus time from exposure; Panel B displays the breadth of the HIV-specific responses as measured by the percent of HIV peptides tested able to stimulate a positive response versus time from exposure. In Panel A the number spot forming cells (SFC) is the sum of each individual peptide-specific response that generated results considered to be above background. In panel B the percent of reactive peptides refers to the number of peptides generating a positive response divided by the number of peptides tested.

Figure 3. Proportion of HIV peptides tested stimulating a positive response displayed by group. Panel A shows results for all HIV peptides tested. Panels B, C, D and E show results for Env-, Gag-, RT-, and Nef-derived HIV peptides, respectively.

Figure 4. Reactivity to HLA-A*0201 restricted peptides Gag 77-85 and RT 476-484 in subjects in PI and chronic infection. Seven HLA-A*0201 positive subjects in early PI, 4 in late PI and 13 in the chronic phase of infection were screened for reactivity to HLA-A*0201 restricted Gag 77-85 (Panel A) and RT 476-484 (Panel B) by the IFN-γ Elispot assay. SFC/million PBMC refers to the number of spot forming cells (IFN-γ secreting cells) generated per million peripheral blood mononuclear cells following stimulation with each peptide. Reactivity to no peptides has been subtracted from the results displayed in the graph.

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Chapter 3: Longitudinal Analysis of HIV-Specific Effector Responses in Patients Initiating HAART in HIV PI

In chapter 2 cross sectional data comparing HIV-specific immune responses in treatment naïve HIV infected individuals at various times from infection was evaluated. Despite limitations inherent in cross sectional studies, the work reveals that the HIV-specific immune response expanded both in breadth and magnitude during the first two months of infection and that Nef appeared to be preferentially targeted at this time.

At the time the Quebec PI cohort study population was recruited the standard of care was to offer HAART to all newly infected individuals. HAART has the ability to rescue or preserve HIV-specific CD4+ T cell activity in acute infection. Maintenance of these responses may permit HIV infected individuals to control HIV as do long term non progressor who also maintain their HIV-specific T cell function. The hope is that HIV-specific CD4+ T cells will make the immune system better able to respond to immunotherapeutic strategies, vaccines, or treatment interruptions.

Access to longitudinal samples collected from individuals starting HAART at various times in PI provided the impetus for the investigations presented in chapter 3. Here we address the potential "immune-preservative" role of therapy in patients receiving therapy beyond acute infection, thus to assess how long from infection could initiation of HAART be delayed without loosing its immunological reconstitutive effects.

LONGITUDINAL ASSESSMENT OF CHANGES IN HIV-SPECIFIC EFFECTOR ACTIVITY IN HIV INFECTED PATIENTS STARTING HAART IN PRIMARY INFECTION

Running Title: Longitudinal HIV-specific responses in primary HIV infection

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3.1 Abstract

Both the magnitude and breadth of HIV-specific immunity was evaluated longitudinally on samples collected from 6 subjects starting highly active antiretroviral therapy (HAART) pre-seroconversion (group 1), 11 recently infected subjects starting HAART post-seroconversion (group 2), 5 subjects starting HAART in the second half of the first year of infection (group 3) and 6 persons starting treatment in the chronic phase of infection (group 4). HIV-specific immunity was measured by Interferon-γ ELIspot, detecting the frequency of cells responding to a panel of HLA restricted HIV-1 peptides; intracellular cytokine staining was used to detect the frequency of HIV-1 Gag p55 specific CD4+ and CD8+ T cells in a subset of participants. The magnitude and breadth of HIV-specific responses persisted in all group 1 and in 5 of 11 (45%) group 2 subjects. Both these parameters declined in 6 of 11 (55%) group 2 and all group 3 and 4 individuals. All persons who maintained detectable numbers of HIV-1 Gag p55 specific CD4+ and CD8+ T cells after starting HAART preserved the intensity and breadth of their HIV-specific effector response. Our results show that HIV-specific immunity can be preserved even if HAART is initiated beyond the acute phase of infection.

3.2 Introduction

High levels of viral replication followed by induction of an immune response are characteristic features of HIV-1 primary infection (PI) [1-4]. HIV-specific CD8+ cytotoxic T lymphocytes (CTL) are first detected during PI and are believed to play a key role in controlling viral replication at this time [3-6]. The type of immune response induced during PI appears to determine viral set point and the subsequent course of infection [2;7-9]. Despite the development of HIV-specific immunity in PI, these responses are usually ineffective at eradicating the virus and disease progresses without treatment. A subpopulation of HIV-infected individuals termed long-term non-progressors has a more benign course of disease. They differ from most other HIV-infected individuals by preserving HIV-specific CD4+ activity and strong and broad CD8+ virus-specific T cell responses [10-12]. These observations have given impetus to finding treatment regimens that favor the preservation of HIV-specific immunity in the hopes that such responses will better control viral replication [9].

Highly active antiretroviral therapy (HAART) including at least 2 reverse transcriptase inhibitors (RTI) and at least one protease inhibitor or non-nucleoside transcriptase inhibitor (NNRTI) can suppress the replication of HIV in most anti-retroviral therapy naïve patients adhering to their drug regimens [13;14]. Initiating HAART is recommended for acute HIV-1 infection to reduce viral dissemination and its harmful effects on the immune system [15]. Suppressive HAART started in acute infection preserves or rescues HIV-specific CD4+ T cell function [9;11;16;17]. At this stage of infection the breadth (number of HIV peptides recognized) and magnitude (The frequency of HIV specific cells) of the HIV-specific CD8+ T cell response is significantly less than that

observed later in infection [16;18;19]. HIV-specific effector function, that is present, persists but does not appear to expand after starting HAART [16;17]. One report showed that HIV-specific T helper activity was preserved when HAART was started as late as 137 days from HIV acquisition [20]. Based on these findings we hypothesized that there was a window beyond the acute seroconversion phase within which starting HAART would permit the preservation of a fully developed HIV-effector response due to persistence of HIV-specific T helper activity.

We present here the results of a longitudinal study comparing changes in the breadth and magnitude of CD8+ T cell effector responses in 4 groups of treatment naïve subjects following introduction of successful HAART that suppressed viral load to below 50 copies/ml of plasma. The study groups differed from each other in the interval elapsed between infection and start of HAART. Subjects in group 1 (n=6) started HAART pre-seroconversion. Subjects in group 2 (n=11) began HAART after seroconversion but within approximately 6 months of infection. Group 3 (n=5) included individuals who started therapy later in the first year of infection whereas subjects in group 4 (n=6) began treatment during the chronic phase of infection. Here, using a quantitative HIV-peptide specific Interferon-γ (IFN-γ) enzyme linked immunospot (ELIspot) assay, we show that all subjects in group 1, and 5 of 11 subjects in group 2, maintained the breadth and magnitude of HIV-specific effector activity after starting HAART. In contrast, HIV-specific effector activity declined in 6 of 11 subjects from group 2 as well as in those from groups 3 and 4. All individuals in groups 1 and 2, who maintained detectable levels of HIV-1 Gag p55-specific responses in both their CD4+ and CD8+ T cell compartments as evidenced by intracellular cytokine staining assays, also preserved the intensity and

breadth of their HIV effector response. The persons in groups 2, 3 and 4 whose HIV-1 Gag p55-specific CD4+ and CD8+ T cells number fell below background levels also exhibited a decline in the intensity and a narrowing in the breadth of their HIV-specific effector response after starting HAART.

3.3 Methods

Study population: We studied 22 subjects enrolled in the Quebec HIV Primary Infection cohort and 6 subjects in the chronic phase of infection. The institutional review boards of all participating study sites approved the study and all participants signed informed consent. All subjects were anti-retroviral drug naïve at study entry. Subjects in the Quebec PI cohort were followed clinically at study entry, at week 2, 4, 6, 8 and every 3 months until month 24. Subjects in chronic infection were followed monthly for 24 months. All study participants, with the exception of MQPI018 and MQPI020, started HAART consisting of at least two RTI and at least 1 Protease Inhibitor or NNRTI at the first clinic visit. MQPI018 started HAART at visit 5 (2 months after study entry), and MQPI020 started HAART at visit 8 (9 months after study entry). The date HAART was started was defined as baseline for the results reported here.

Entry criteria for the Quebec HIV Primary Infection cohort have been described previously [19]. The presumed date of infection was estimated for each individual using clinical and laboratory data as well as patient history information. The following guidelines proposed by the Acute HIV Infection and Early Disease Research Program sponsored by the National Institutes of Allergy and Infectious Disease Division of AIDS were used estimate the date of infection: the date of the first indeterminate Western blot minus 35 days; the date of a positive HIV RNA test or p24 antigen assay available on the same day as a negative HIV EIA test minus 14 days. The date of onset of symptoms of an acute retroviral syndrome minus 14 days was also used to estimate the date of infection [18]. Information obtained from questionnaires addressing the timing of high-risk behavior for HIV transmission was used when available to confirm the presumed date of exposure.

Participants were classified into study groups based on the time elapsed between infection and start of HAART. Subjects in group 1 (n=6) started HAART before seroconversion as defined by a negative or indeterminate standard HIV-1 antibody enzyme immunoassay (EIA) and confirmatory Western blot. They were also negative in a less sensitive HIV-1 antibody EIA (LS-EIA). This assay can distinguish recent HIV infection from chronic infection [21]. The cut off for the LS-EIA used to classify participants into groups for this study was 1.0, which identifies individuals as infected less than 170 days (95% confidence intervals 162-183 days). Subjects were classified as belonging to group 2 (n=11) if they began HAART at a time when they had positive results using in the standard EIA but were negative using the LS-EIA. Subjects classified as group 3 (n=5) were positive in both the standard and LS-EIA. For comparison group 4 included 6 asymptomatic chronically infected subjects who were seropositive a median of 4.4 (range 2-14) yrs.

Laboratory testing: HIV-1 antibody testing was performed at three University hospital sites. The LS-EIA was carried out at the University of California, San Francisco, CA using the Vironostica HIV-1 EIA (Organon-Tecnika, Boxtel, the Netherlands). Western blot analysis for HIV-1 antibodies and p24 antigen capture assays were performed at the Laboratoire de Santé Publique du Québec. Plasma viremia was measured using the Roche Amplicor Assay (Roche Diagnostics, Mississauga, Ontario, Canada) with detection limit of 500 HIV-1 RNA copies/ml of plasma. Plasma samples falling below the detection limit of this assay were retested using the ultrasensitive method (Ultradirect, Roche) with a detection limit of 50 copies/ml of plasma. T cell subset distribution was measured by flow cytometric analysis.

Cells and peptides: PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) and frozen in 10% dimethyl sulfoxide (DMSO, Sigma, St Louis MO) in 90% fetal calf serum (FCS, Montreal Biotech Inc., Montreal, QC, Canada). Subjects were typed for major histocompatibility complex (MHC) class I antigen expression by the amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles [22] (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI). Genomic DNA for molecular HLA-typing was prepared from either fresh blood or Epstein-Barr virus (EBV) transformed B cell lines using the QIAamp DNA blood kit (Qiagen Inc., Mississauga, ON).

Peptide Selection: The HIV epitopes used for stimulation were chosen from the National Institutes of Health (NIH) HIV molecular immunology database [23]. Peptides of 9-, 15-, or 20-aa in length containing these sequences were obtained from the Medical Research Council AIDS Reagent Project (Hertz, UK) and the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Lyophilized peptides were diluted to 1 mg/ml in Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY) containing 10% DMSO and stored at -70°C. They were used at a final concentration of 10 μM (20 to 40 ug/ml, depending on the peptide size).

Elispot assay for single cell IFN-γ release: IFN-γ secretion by virus-specific cells was quantified by ELIspot assay as described [19]. Stimulating peptides were chosen on the basis of their having amino acid sequence binding motifs for the MHC class I alleles expressed by the person being tested. Stimulatory panels contained peptides restricted to multiple (2 to 5) MHC class I alleles per subject. Media, containing the

equivalent amount of DMSO as present in peptide stimulation conditions, was used as a negative control. Anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) was used as a positive control stimulus. Cells were plated at two concentrations ($2x10^5$ cells/well and $5x10^4$ cells/well) for each peptide condition. For a subset of experiments a pool of immunodominant HLA-A2- or HLA-B7-restricted EBV- and Cytomegalovirus- (CMV) derived peptides was also used as positive control stimuli. The frequency of reactivity to anti-CD3 and the EBV/CMV peptide pool stimuli occurring in longitudinally collected samples was used to control for between-time point variability in cell responsiveness. Results are expressed as spot forming cells (SFC)/10^6 PBMC following subtraction of negative controls. Negative control stimulation produced less than 5 spots per well in greater than 90% of experiments. The average number of spots in the negative control wells was 3.13 ± 3.12. In experimental wells the signal was considered positive if at least 10 spots per well were present (>2 standard deviation above the mean), the number of spots obtained was proportional to the number of cells plated and the number of spots per well that was at least 2-fold greater than the negative control wells. The identity of IFN-γ secreting cells as CD8+ was confirmed by the reduction of SFC numbers following depletion of CD8+ cells with magnetic beads (Dynal, Lake Success, NY).

Intracellular Cytokine Staining: The frequency of Gag p55-specific CD4+ and CD8+ T cells was quantitated by intracellular cytokine staining. Frozen and thawed PBMC were resuspended at 10^6 cells/ml in RPMI 1640 containing 10% FCS, 2 mM L-glutamine (ICN Biomedical Canada Ltd., Costa Mesa, CA), 50 IU/ml penicillin (ICN), 50 mcg/ml streptomycin (ICN) and 50 μM 2-mercaptoethanol (Sigma) and stimulated with a pool of 122 HIV 15-mer peptides with 11 amino acid overlaps corresponding to

the HIV-1 HXB2 Gag p55 sequence (NIH AIDS Research and Reference Reagent Program). Staphylococcal enterotoxin B (SEB; Sigma) was employed as the positive control; medium alone served as the negative control. Cells were incubated for 2 hr at 37°C in 5% CO_2 with stimulatory peptides and 1 μg/ml each of anti-CD28 and anti-CD49d monoclonal antibodies (mAbs) (BD Biosciences, Mississauga, ON). Brefeldin A (Sigma) was added at a final concentration of 10 μg/mL and incubated for an additional 4 hours at 37°C in 5% CO_2. Cells were permeabilized with FacsPerm according to manufacturer's direction (BD Biosciences). IFN-γ secretion by recently stimulated CD4+ and CD8+ T cells was detected by staining samples with fluorescein isothiocyamate (FITC)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD69, peridinin cholophyll protein (PerCP)-conjugated anti-IFN-γ mAbs (BD Biosciences) and allophycocyanin (APC) conjugated anti-CD8 (BD Biosciences) for 30 minutes in the dark. In parallel, control samples were left unstained and stained with immunmoglobulin isotype control antibodies (BD Biosciences). After washing cells were resuspended in 1% paraformaldehyde (Polysciences, Inc., Warrington, PA) until four-color flow cytometric analysis was performed on a FACSCalibur instrument (BD Biosciences). Fifty thousand to 200,000 events were acquired and analyzed using Cellquest software. A response was considered positive if the frequency of CD69+INF-γ+CD4+ or CD69+IFN-γ+CD8+ cells present following an antigenic stimulation was greater than 0.1% over that seen with cells stimulated with no antigen.

Statistical Analysis: Two tailed unpaired t-tests were used to assess between-group differences in age, CD4 count, CD8 count, viral load at study entry as well as between group differences in the number of HIV peptides used to screen for HIV-specific

responses. Wilcoxon matched pairs tests were employed to assess within group differences at study entry versus on-therapy time points. Non-parametric Wald-Wolfowitz tests with adjusted z-score and corresponding adjusted p-value for small samples size were used to evaluate the significance of between group differences in the magnitude of HIV-specific responses. Fisher P-exact tests were utilized to test the significance of within- and between -group differences in the breadth of HIV responses. The Fisher P-exact test with Bonferonni correction was used to assess between group differences in the expression of common HLA alleles. P-values of less then 0.05 were considered significant.

3.4 Results

Study population: A description of the study population is provided in Table 1. Subjects in PI were separated into 3 groups based the interval between the presumed date of infection and start of HAART as described in the methods section. There were no differences between groups with respect to age, ratio of males to females, or their distribution in risk behavior categories. All study groups had similar CD4+ and CD8+ T cell counts at the time HAART was started and were followed over a similar period of time (Table 1). Viral load at entry was higher for the individuals in group 1 compared with those in groups 2 and 4. This is consistent with their being closer to acute infection at a time when viremia peaks prior to the establishment of a viral load set point [1-4].

Table 2 provides information for each individual on the composition of HAART prescribed, the classification of each individual into the study groups described in the methods section, estimated date of infection, the clinical information and laboratory results used to derive an estimated date of infection, and expression of MHC class I alleles. The composition of the HIV peptide panels used to test individual subjects for HIV specific responses was dependent on the MHC class I alleles expressed by the subject being tested. For this reason the distribution of common MHC class I alleles such as HLA-A1, A2, A3, A24, B7, B8, B35 and B44 between groups was compared. No significant skewing in the distribution of these alleles was evident between the groups in PI (p= n.s., Fisher P-exact, with Bonferronni correction); nor were between group differences detected with respect to the distribution of alleles likely to be homozygous. Although all individuals in group 4 expressed HLA-A2 the inclusion of HIV peptides restricted to between 2 and 5 alleles per study subjects in each peptide screening panel

makes the possibility that the results reported are due to skewed distribution of MHC class I alleles between groups unlikely.

For many of the within- and between-group comparisons an on-therapy time point was examined. The time from infection to which this on-therapy sample corresponds was the available time point closest to 12 months from the start of treatment. This time point was a median of 348 (range 184-429) days from start of HAART for group 1, 349 (range 84-385) days for group 2, 189 (range 100-357) days for group 3, and 350 (range 266-362) days for group 4. The time used as the on-therapy time point was not significantly different between groups (p=n.s., unpaired t-tests). Furthermore decline in the breadth and magnitude of HIV-specific responses in study subjects in which this occurred after starting HAART was usually evident within 2 months and corresponded to control of viremia.

HIV Specific Effector Responses: Panels of MHC class I restricted HIV peptides were used to stimulate PBMC samples collected longitudinally from the time HAART was initiated. Table 3 lists the sequence and the MHC class I restriction specificity of peptides used in this analysis together with their sequence, location and MHC class I restriction specificity. For each peptide stimulus the frequency of IFN-γ secreting cells generated per 10^6 PBMC was assessed in an ELIspot assay. The magnitude of the response to the HIV peptide panel tested was measured by adding the number of SFC/10^6 PBMC generated to each stimulus that induced above background responses. The breadth of the responses was measured by calculating the number of positive responses in each peptide panel divided by the total number of peptides tested. Longitudinal changes in these parameters were assessed.

Figure 1A shows the changes in the magnitude and breadth of responses to an HIV peptide panel used to screen 1 subject from group 1. Subject MQPI005 began HAART 36 days from infection at which time the subject was positive in a standard EIA but indeterminate in a confirmatory Western blot test and negative in a LS-EIA. This representative group 1 individual maintained the magnitude and the breadth of his response to the HIV peptide panel tested for up to 684 days on therapy, despite suppression of viremia soon after starting HAART. The 5 other members of group 1 similarly maintained or increased the magnitude and breadth of their HIV-1 response to the peptides tested throughout the follow up period of 184, 358, 518, 535, and 630 days from the start of HAART that successfully controlled viral load. A within group comparison of the magnitude and breadth of responses at baseline versus those at the on-therapy time point closest to 12 months revealed a similar breadth and magnitude on therapy versus that at baseline (Table 4).

Figure 1B-L display the longitudinal assessments of reactivity to panels of MHC class I restricted peptides for all 11 individuals belonging to group 2 who began HAART early during infection but after seroconversion, as defined by their being negative in the LS-EIA. Therapy was associated with reduced viremia in this group as well. Two distinct patterns of change in HIV-specific responses were observed in this group after the start of HAART. Figure 1B-F show results for 5 subjects who maintained responses over a follow up of 230 to 815 days. Figure 1G-L show results from all 6 individuals belonging to group 2 whose HIV-specific responses fell in intensity and narrowed in specificity after HAART was initiated and viral load controlled. This decline occurred despite the maintenance of a stable frequency of cells specific for the EBV derived HLA-B7

restricted peptide RPPIFIRRL (Fig 1I) and the EBV derived HLA-B8 restricted peptide FLRGRAYGL (Fig 1J) [24]. Comparison of the intensity and breadth of HIV-peptide panel specific responses at baseline to those at the time point closest to 12 months on-therapy for subjects in group 2 showed a non statistically significant trend towards reduction in the magnitude of the response ($p=0.7$, Wilcoxon matched pairs test) while the breadth of the response fell significantly from baseline to the on-therapy time point ($p=0.048$, Fisher P-exact test) (Table 4).

Group 2 subjects who maintained their HIV-specific responses started HAART a median of 97 (range 69 to 138) days from infection whereas those who lost these responses began treatment a median 134 (range 72 to 173) days from infection. Although there was a tendency to maintain persistent effector responses after starting therapy in subjects treated at earlier versus later times after infection and seroconversion this differences was not statistically significant ($p=0.27$; unpaired t-test). No differences were detected in the viral load at therapy initiation between the subgroup that maintained versus the one that lost HIV-specific effector activity.

Retrospective statistical analysis of group 2, categorized by whether they maintained (group 2a) or lost (group 2b) HIV-specific effector responses, revealed that responses were as intense and broad in subgroup 2a following one year of therapy as they were prior to starting HAART. In contrast, for subjects belonging to group 2b, the breadth of this activity at the closest available time tested to 12 months on-therapy was lower than at baseline ($p<0.02$, Fisher P-exact test). The magnitude of HIV-specific reactivity showed a non-significant trend towards a reduced response ($p=0.07$, Wilcoxon matched pairs test) (Table 4).

Figure 1M and N display longitudinal analyses of HIV-specific reactivity for a group 3 (Fig 1M) and for a group 4 subject (Fig 1N). As has been described by others, persons starting HAART in the chronic phase of infection displayed reduced magnitude and breadth of HIV-specific responses following start of HAART in association with viral load suppression [25-28]. Possibly due to small group size the reduction in the magnitude and breadth of the HIV responses from baseline to the closest available time point to 12 months on-therapy was only statistically significant for breadth for group 3 ($p<0.001$, χ^2-test) (Table 4). No differences were detected for comparisons of the breadth and magnitude of HIV peptide panel specific responses at baseline and 12 months between groups 3 and 4. We therefore combined the study subjects in groups 3 and 4 into a single data set and assessed the significance of changes in breadth and magnitude of HIV-specific responses at baseline versus the on-therapy time points. For both measures responses were reduced on-therapy compared to baseline ($p=0.02$, Wilcoxon matched pairs test; $p<0.001$, Fisher P-exact test).

All of the subjects in the PI study groups were re-categorized according to whether they maintained or lost HIV-specific responses and compared for within- and between-group differences in HIV specific effector responses. To determine whether viral load could account for whether an individual maintained or lost of HIV specific immune responses in this population we compared viral load at baseline. No differences were detected between subjects who maintained (groups 1 + 2a) versus those who lost (group 2b + 3) HIV-specific responses after starting HAART (Fig. 2A). Comparisons of the breadth of responses to HIV peptide panels showed maintenance of the reactivity from baseline to the on-therapy time point for group 1 + 2a (p=n.s., Fisher P=exact test)

and a decline in responsiveness for group 2b + 3 ($p<0.001$, Fisher P-exact test) (Fig 2B). HIV-specific responses in these 2 groups were similar at baseline but significantly narrower at the time point closest to 12 months on-therapy for group 2b and 3 versus group 1 and 2a ($p<0.001$, Fisher P-exact test) (Fig 2B). Comparison of the magnitude also showed maintenance of the reactivity from baseline to the on-therapy time point for group 1 + 2a (p=n.s., Wilcoxon matched pairs test) and a decline in responsiveness for group 2b + 3 ($p<0.03$, Wilcoxon matched pairs test) (Fig 2C). HIV-specific reactivity appeared to be greater prior to starting HAART for subjects in groups 1 + 2a compared to those for group 2b + 3 but this difference was not statistically significant (Fig 2C). At the on-therapy time point between-group comparisons revealed that subjects in group 1 + 2a had a non statistically significant trend towards having a more intense response to the peptide panel tested than did subjects in group 2b + 3 ($p=0.08$, Wald-Wolfowitz with adjusted z-score) (Fig 2C).

Starting HAART in the acute phase of HIV infection has been shown to maintain HIV-specific CD4+ T cells, which are preferred targets for HIV infection [9;11;17]. We reasoned, based on the observation that HIV-specific effector responses could be preserved in some individuals starting HAART after seroconversion, that this reflected the preservation of functional HIV-specific CD4+ T cell levels. Detection of intracellular INF-γ production in the CD4+ and CD8+ compartments following stimulation with a pool of HIV-1 Gag p55 peptides was used to screen longitudinal samples from a subset of subjects from each group. All subjects tested from group 1 (24, 31, 54, and 58 days from presumed date of infection) maintained HIV specific activity in both compartments up to 12 months from start of HAART (Figure 3B and C). Seven 7 subjects from group 2 were

tested in this manner. Three individuals classified as belonging to group 2a (MQPI001, MQPI009, MQPI017, 69, 76, and 138 days from infection, respectively) maintained HIV specific activity as measured by ELIspot (Figure 1B, D and F). Four subjects from group 2 (MQPI012, MQPI013, MQPI014 and MQPI016, 83, 135, 146 and 173 days from infection, respectively) lost HIV-specific responses in association with viral control (Figure 1H, I, J and L). By intracellular cytokine staining for IFN-γ HIV Gag p55-specific CD4+ and CD8+ cells persisted in all group 2a subjects (Figure 3B and C). The decline in HIV-specific effector responses in group 2b individuals observed by ELIspot assay was also seen within 3 months of initiating HAART for HIV-1 Gag p55 CD4+ and CD8+ T cells (Figure3 B and C). MQPI018 and MQPI021 from group 3 were estimated to be 272 and 279 days from infection when they began therapy. Both lost HIV specific cell numbers in both the CD4+ and CD8+ T cell compartments as early as 2 months from start of HAART (Figure 3 B and C). The two subjects tested from group 4 (CH5 and CH6) also showed a decline in the frequency of their HIV-1 Gag p55-specific CD4+ and CD8+ cells to below background values within one month of initiation of HAART (Figure 3B and C).

3.5 Discussion

HIV-specific CD8+ and CD4+ T cell frequencies were monitored over time using the IFN-γ ELIspot and intracellular cytokine staining assays in subjects starting HAART at various times during the first year of infection. This report shows that all individuals tested, who began HAART before seroconversion and 5 of 11 (45%) of those who started treatment after seroconversion while still negative in a LS-EIA maintained the breadth and magnitude of their HIV-specific effector response over follow up period ranging from 184 to 815 days after starting HAART that suppressed viremia. Four group 1 subjects and three group 2a individuals who maintained HIV-specific effector activity as determined by ELIspot assay, also maintained detectable levels of both CD8+ and CD4+ T cells specific for HIV-1 Gag p55 by intracellular cytokine staining for up to 12 months after starting HAART (Fig 3B and C). HIV-specific immunity in 6 of 11 (55%) seropositive subjects starting HAART at a time when they were negative by LS-EIA and in all subjects who began therapy when seropositive by both the standard and LS-EIA had a similar outcome to that in persons starting HAART in chronic infection. The magnitude and breadth of the response to the HIV peptide panel with which they were tested declined significantly from baseline values [25-28]. Furthermore, the frequency of CD8+ and CD4+ T cells responding to HIV-1 Gag p55 fell to below background levels by 3 months after starting HAART in all the individuals tested from groups 2b, 3 and 4 who exhibited declining HIV-specific effector responses by ELIspot (Fig 3B and C).

The Los Alamos Molecular Immunology database was used to select the HLA-restricted peptides included in stimulatory peptide panels. This database was compiled predominantly from data generated in subjects in the chronic phase of HIV infection [29].

As the immunodominance pattern of HIV epitope recognition may differ with disease stage it is possible that subjects in PI would have poorer reactivity to peptide panels assembled in this manner [16;30]. Using a larger population of HIV infected patient population in PI, we confirmed the work of others by showing that HAART naïve subjects within 2 months of HIV infection have a significantly reduced breadth and magnitude of HIV-specific CD8+ T cell reactivity compared to individuals tested between 2 and 4 months of infection or later in the first year of infection [16;18;19;31]. The pre-seroconversion PI group (group 1) studied here did not have HIV responses of a lower magnitude than those starting HAART later in PI. The most likely explanation for failing to see a narrower and less intense HIV-specific response for group 1 is the small sample size. An alternate explanation may be that group 1 subjects, although pre-seroconversion, were at a later stage of PI than the population described by Altfeld et al. such that they had the opportunity to develop broader responses than those seen in subjects an earlier or acute phase of HIV infection [16;31]. Group 1 was estimated to be a median of 32 days from infection (range 21 to 58 days), which was at an earlier stage of infection than that studied by Dalod et al who also observed a more restricted HIV-specific response in their PI cohort [18]. If results from subjects in group 1 were combined with those in group 2a and compared with those from subjects later in infection (group 2b and 3) a non significant trend towards a more intense responses was observed in the individuals who were more recently infected (Fig 2C).

The concentration of peptide used to stimulate PBMC from study subjects was approximately 10-fold higher than that used by others[32-34]. We performed some experiments to control for the possibility that between group differences in longitudinal

peptide reactivity in PBMC from subjects starting HAART at different times after infection are differentially susceptible to activation induced cell death (AICD) associated with high peptide concentrations. PBMC samples from multiple time points spanning the first year on HAART were stimulated with a titration series of several stimulatory peptides. Four serial 10-fold dilutions were tested starting with the same concentration of peptide used in the original experiments. A subset of subjects from each study group was screened with 3 to 4 stimulatory peptides each. All peptide/PBMC combinations that generated positive responses in the first experiment were positive in the titration experiment. All stimulatory peptides generated the same number of SFC/10^6 PBMC at a dilution of at least 1:100 as they did at the concentration used in the initial experiments (not shown). Therefore between-group differences in susceptibility to AICD do not appear to play a role in the ELIspot results presented here using peptide concentrations of 10 uM as stimuli.

All of the individuals in groups 1 and 2 who developed persistent HIV-specific responses maintained not only the magnitude and breadth of their HIV specific response but also the hierarchy of their pre-therapy responses in terms of the intensity of reactivity to individual peptides for the entire follow up period. This observation contrasts with that seen in subjects who lost responses after starting HAART. The pattern of loss was similar in the subset of group 2 subjects with declining responses (group 2b) as it was for group 3 and 4 subjects (Figure 1G-N). The timing after infection at which responses could no longer be maintained after starting therapy appeared to be subject to biological variation but occurred after seroconversion and in all individuals tested who were 146 days and beyond infection. The biological variability is evident by contrasting MQPI011 and

MQPI013 who lost responses even through HAART was begun as early as 73 days and 83 days from estimated date of infection with MQPI017 who maintained responses when HAART was begun 138 days from estimated date of infection. In all three of these individuals information on the date on which symptoms of acute infection appeared or a dated indeterminate Western blot was available to estimate the timing of infection. While it is possible that MQPI011 and MQPI013 were infected earlier if the interval between infection and appearance of symptoms was longer than 14 days, and MQPI017 was infected later if the indeterminate Western blot test result reflected infection for longer than 35 days, it is unlikely that MQPI017 started HAART sooner after infection that MQPI011 and MQPI013.

The persistence of effector responses for patients on HAART appeared to be related to the ability to rescue or maintain HIV-specific CD4+ responses. The timing of the "point of no return" in terms of the potential to rescue HIV-specific responses is consistent with data generated by Malhotra et al. using proliferation to HIV-1 Gag p24 as a measure of HIV-specific CD4+ T cell responses [20;35]. These authors reported that HAART started as late as 137 days after infection could rescue HIV-1 Gag p24 proliferation responses. [20;35].

The implication of the results presented here is that preservation of HIV-specific CD4+ and CD8+ responses is not limited to situations where HAART can be started during the acute phase of infection at a time when HIV-specific CD8+ T cell immunity appears to be reduced compared to that seen at later times in PI [16;18;19]. Delaying HAART initiation beyond the acute phase of infection may allow the breadth and magnitude of HIV-specific CD8+ T cell reactivity to develop to its full potential[19] without losing the

advantage of being able to preserve or rescue HIV-specific CD4+ T cell responses [9;11;16;17;20;35]. Due to uncertainty as to when a particular individual will reach a stage in disease progression when HIV-specific immunity no longer persists on treatment underlines the advantage to starting therapy as soon as possible after infection in individuals who are negative in the LS-EIA.

High levels of HIV viremia appear to be required to drive the persistence of the HIV specific responses in subjects in late PI and the chronic phase of infection. If antigen load were the only explanation for elevated levels of virus specific immune cells, then one would assume that successfully treated subjects in early PI would also exhibit a decline in the frequency of HIV-specific reactivity. The maintenance of these responses in subjects initiating HAART in early PI is therefore likely due to the persistence of HIV-specific T helper activity. In the presence of T cell help low levels of viremia are sufficient to maintain a potent and broad effector response to HIV. This situation may be analogous to that observed in long term non-progressors where HIV-specific T helper responses are also preserved and HIV-specific effector activity is maintained [9;11]. This phenomenon would be consistent with mouse models of LCMV infection where virus-specific T helper cells are required for persistence of CD8+ CTL function beyond the acute phase of infection and for establishment of functional LCMV-specific memory [36-39].

The individuals studied here have not been followed long enough to appreciate the clinical implications of preserving HIV-specific immunity by introduction of HAART early in PI but beyond seroconversion. Rosenberg et al. have demonstrated that a proportion of persons who initiated HAART during acute infection were able to control viremia after treatment was interrupted for the first time [9;40]. This proportion increased

with subsequent cycles of therapy withdrawal [9;40]. Preservation of HIV-specific CD4+ and CD8+ T cells function by starting HAART in early PI but after the acute phase of infection may mediate 2 alternative outcomes. Maintenance of HIV-specific immunity may be sufficient to control viremia upon treatment withdrawal in a similar fashion to that seen when HAART is begun during acute infection [16;40]. Alternately, the delay in initiation of HAART may result in the loss of the T cell clones best able to control virus. In macaques infected with simian immunodeficiency virus, CTL escape mutations occur within weeks of infection [41]. If loss of the clones best able to control infection occurs early in HIV-1 infection, delaying HAART may compromise the ability to control virus when therapy is withdrawn even though a strong and broad HIV-specific immune response is present.

The results presented here support the conclusion that aggressive HAART before and in some cases soon after seroconversion permits the maintenance of HIV-specific CD4+ T helper and CD8 effector responses. Knowing whether therapy initiation within this interval has a clinical benefit in terms of preserving HIV-specific immunity able to control viremia upon HAART withdrawal similar to what has been reported for treatment during acute infection is important information for HIV-1 infected patient care. The acute HIV infection syndrome is non-specific, variable and does not occur in all individuals who become HIV-1 infected [42-47]. As a result, symptoms of acute seroconversion can go unrecognized as due to early HIV infection, thereby resulting in delays in the start treatment. Increasing the window within which successful HAART can mediate a benefit on HIV-specific immune system preservation and clinical outcome to times soon after

seroconversion would provide supporting data that a greater number of newly HIV-1 infected subjects can be treated optimally.

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Figure 1

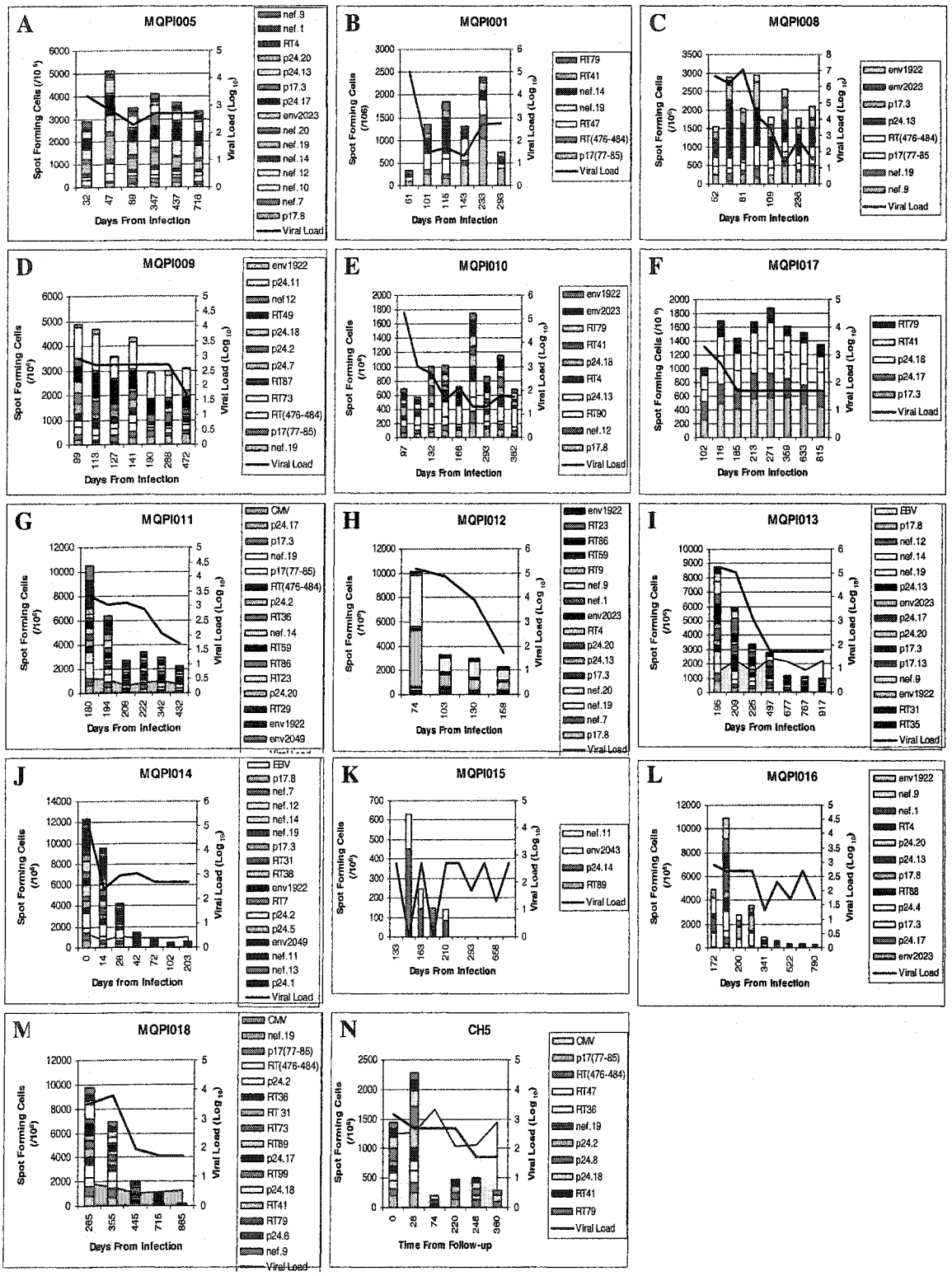

A
MQPI005
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
nef.9
nef.1
RT4
p24.20
p24.13
p17.3
p24.17
env2023
nef.20
nef.19
nef.14
nef.12
nef.10
nef.7
p17.8
Viral Load
B
MQPI001
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
RT79
RT41
nef.14
nef.19
RT47
RT(476-484)
p17(77-85)
Viral Load
C
MQPI008
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
env1922
env2023
p17.3
p24.13
RT(476-484)
p17(77-85)
nef.19
nef.9
Viral Load
D
MQPI009
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
env1922
p24.11
nef12
RT49
p24.18
p24.2
p24.7
RT87
RT73
RT(476-484)
p17(77-85)
nef.19
Viral Load
E
MQPI010
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
env1922
env2023
RT79
RT41
p24.18
RT4
p24.13
RT90
nef.12
p17.8
Viral Load
F
MQPI017
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
RT79
RT41
p24.18
p24.17
p17.3
Viral Load
G
MQPI011
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
CMV
p24.17
p17.3
nef.19
p17(77-85)
RT(476-484)
p24.2
RT36
nef.14
RT59
RT86
RT23
p24.20
RT29
env1922
env2049
H
MQPI012
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
env1922
RT23
RT86
RT59
RT9
nef.9
nef.1
env2023
RT4
p24.20
p24.13
p17.3
nef.20
nef.19
nef.7
p17.8
Viral Load
I
MQPI013
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
EBV
p17.8
nef.12
nef.14
nef.19
p24.13
env2023
p24.17
p24.20
p17.3
p17.13
nef.9
env1922
RT31
RT35
Viral Load
J
MQPI014
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days from Infection
EBV
p17.8
nef.7
nef.12
nef.14
nef.19
p17.3
RT31
RT38
env1922
RT7
p24.2
p24.5
env2049
nef.11
nef.13
p24.1
Viral Load
K
MQPI015
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
nef.11
env2043
p24.14
RT89
Viral Load
L
MQPI016
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
env1922
nef.9
nef.1
RT4
p24.20
p24.13
p17.8
RT88
p24.4
p17.3
p24.17
env2023
Viral Load
M
MQPI018
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Days From Infection
CMV
nef.19
p17(77-85)
RT(476-484)
p24.2
RT36
RT 31
RT73
RT89
p24.17
RT99
p24.18
RT41
RT79
p24.6
nef.9
Viral Load
N
CH5
Spot Forming Cells (/10 6)
Viral Load (Log 10)
Time From Follow-up
CMV
p17(77-85)
RT(476-484)
RT47
RT36
nef.19
p24.2
p24.8
p24.18
RT41
RT79
Viral Load

Figure 2

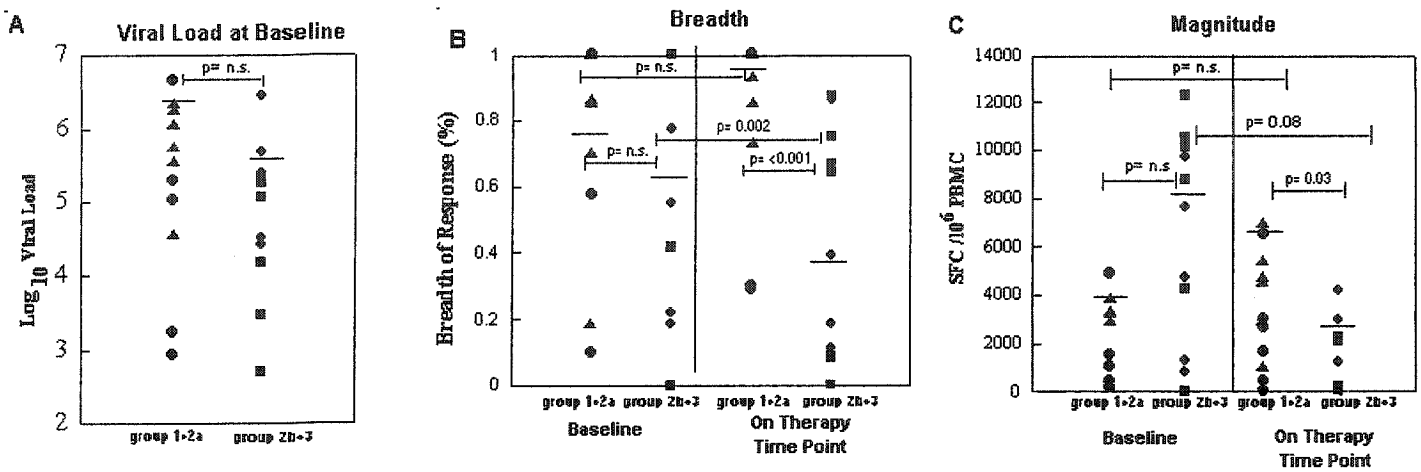
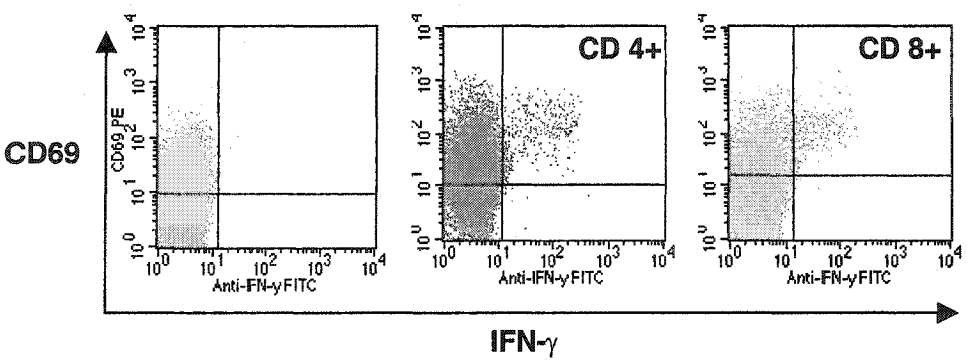
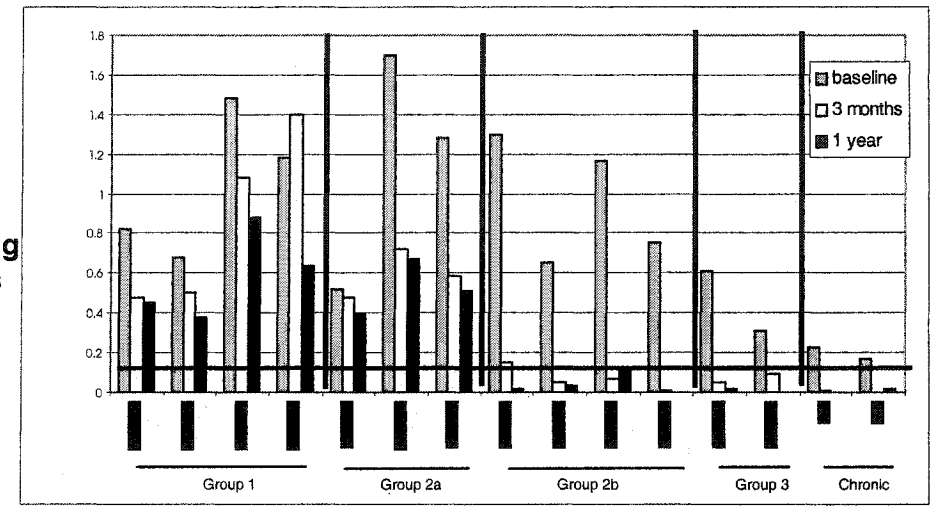

A
Viral Load at Baseline
Log 10 Viral Load
p= n.s.
group 1+2a
group 2b+3
B
Breadth
Breadth of Response (%)
p= n.s.
p= n.s.
p= 0.002
p= <0.001
group 1+2a
group 2b+3
group 1+2a
group 2b+3
Baseline
On Therapy
Time Point
C
Magnitude
SFC /10^6 PBMC
p= n.s.
p= n.s
p= 0.08
p= 0.03
group 1+2a
group 2b+3
group 1+2a
group 2b+3
Baseline
On Therapy
Time Point

Figure 3


CD69
CD69_PE
Anti-IFN-γ FITC
CD 4+
CD 8+
IFN-γ


IFN-γ Secreting CD8+ T cells
baseline
3 months
1 year
Group 1
Group 2a
Group 2b
Group 3
Chronic

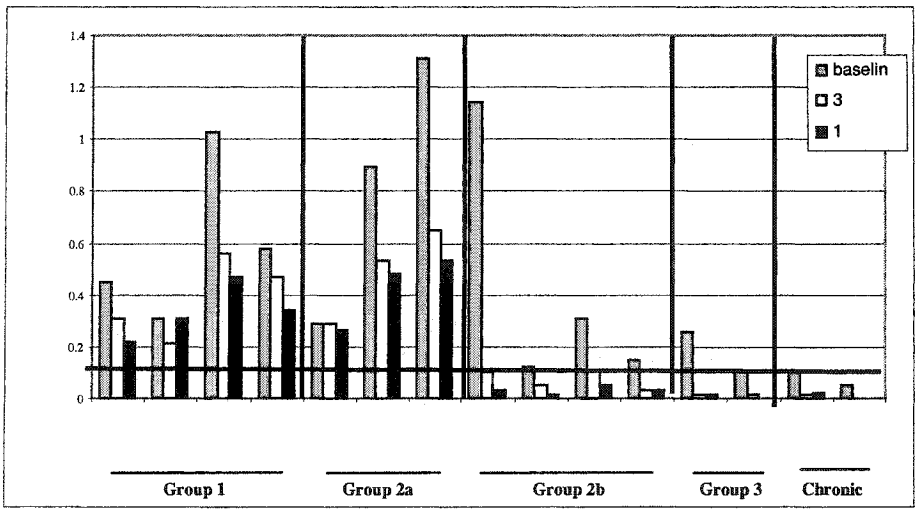

IFN-γ Secreting CD4+ T cells
baselin
3
1
Group 1
Group 2a
Group 2b
Group 3
Chronic

Table 1. Study Population Characteristics at Baseline

		Pre-Seroconversion *(Group 1) (n=6)*	Post-Seroconversion, recently infected *(Group 2)(n=11)*	Post Seroconversion, infected 6 to 12 months *(Group 3) (n=5)*	Chronic *(Group 4) (n=6)*
Age		36[1] (27-42)	41 (29-53)	34 (19-48)	42 (35-55)
Viral Load		5.9 (4.6-6.3)	5.1 (2.7-6.7)	5.4 (4.4-6.4)	3.72 (3.2-5)
CD4		484 (300-1080)	533 (328-1140)	380 (34-918)	423 (264-480)
CD8		1269 (447-4029)	640 (396-4000)	1140 (193-1564)	1072 (575-2070)
Days from Infection		32 (21-58)	100 (69-172)	272 (244-279)	4.4 years (2-14)
Follow-Up		573 (209-716)	428 (203-841)	474 (235-865)	493 (265-563)
Symptoms	Yes	4	8	3	n/a[1]
	No	2	3	2	n/a[1]
Sex	M	6	11	4	7
	F	0	0	1	0
Mode of Transmission	MSM	6	11	3	7
	IVDU	0	0	2	0
Size of Peptide Screening Panels		14.5 (10-19)	14 (4-20)	17 (12-20)	16 (11-24)

[1] *median (range)*
n/a = not available

Table 2.Patient Characterization at Baseline

	Drugs	Group	Estimated Time from Infection	Method used to estimate date of infection	MHC Class I		
					A	B	C
MQPI001	AZT, 3TC, Indinavir	2a	69	1, 4, 5	A2	B62/B44	Cw3/Cw7
MQPI002	Amprenavir, AZT, 3TC, Abacavir	1	21	1, 2, 4	A1/A29	B44/B57	Cw6/Cw16
MQPI003	3TC, d4T, Nelfinavir	1	28	1, 2, 4	A1/A18	B8/B18	Cw5/Cw7
MQPI004	d4T, 3TC, Indinavir	1	58	1, 2, 4, 5	A11/A24	B14/B60	Cw5
MQPI005	AZT, 3TC, Indinavir	1	24	1, 2, 4, 5	A1/A24	B8/B18	Cw5/Cw7
MQPI006	D4T, 3TC, Indinavir	1	36	2, 4, 5	A2/A25	B14/B56	Cw1/Cw3
MQPI007	AZT, 3TC, Indinavir	1	54	2, 4, 5	A2	B18/B44	Cw5/Cw12
MQPI008	AZT, 3TC, Indinavir	2a	97	1, 2, 4, 5	A1/A2	B8	Cw7
MQPI009	d4T, 3TC, Ritonavir, Saquinavir	2a	100	1, 2, 4, 5	A2/A68	B39/B57	Cw7/Cw18
MQPI010	AZT, 3TC, Ritonavir	2a	97	4, 5	A1/A29	B8/B44	Cw16
MQPI011	AZT, 3TC, Ritonavir	2b	73	1, 2, 4, 5	A2/A24	B18/B51	Cw7/Cw15
MQPI012	AZT, 3TC, Indinavir	2b	135	1, 4, 5	A1/A30	B8/B51	Cw7/Cw16
MQPI013	AZT, 3TC, Indinavir	2b	83	1, 4, 5	A1/A24	B8/B35	Cw4/Cw7
MQPI014	AZT, 3TC, Ritonavir	2b	146	1, 4, 5	A1/A3	B7/B39	Cw7/Cw15
MQPI015	AZT, 3TC, Indinavir	2b	133	4, 5	A29/A30	B27	Cw2/Cw6
MQPI016	AZT, 3TC, Indinavir	2b	172	1, 4, 5	A24/A26	B8/B38	Cw7/Cw12
MQPI017	d4T, 3TC, Indinavir, Efavirenz	2a	138	2, 4, 5	A23/A31	B44	Cw4/Cw5
MQPI018	d4T, 3TC, Indinavir	3	272	4, 5	A2/A68	B14/B44	Cw5/Cw8
MQPI019	AZT, 3TC, Indinavir	3	333	4, 5	A11/A24	B35/B49	Cw4/Cw7
MQPI020	D4T, 3TC, Efavirenz	3	253	1, 4, 5	A2/A24	B18/B40	Cw3/Cw12
MQPI021	d4T, 3TC, Indinavir	3	244	4, 5	A3/A68	B7/B14	Cw7/Cw8
MQPI022	AZT, 3TC, Indinavir	3	362	4, 5	A1/A23	B8/B44	Cw4/Cw7
CH1	AZT, 3TC, Saquinavir	4	5 years	n/a	A2/A3	B35	Cw4
CH2	AZT, 3TC, Saquinavir	4	14 years	n/a	A2/A24	B44/B62	Cw3/Cw6
CH3	AZT, 3TC, Saquinavir	4	2 years	n/a	A2/A11	B18/B62	Cw4/Cw7
CH4	AZT, 3TC, Saquinavir	4	4 years	n/a	A2/A24	B62/B44	Cw3/Cw7
CH5	AZT, 3TC, Indinavir	4	5 years	n/a	A2/A25	B44	Cw5
CH7	AZT, 3TC, Indinavir	4	4 years	n/a	A2/A3	B7/B57	Cw7

1 Date of start of symptoms of acute retriviral syndrome -14days
2 Date of first available indeterminate Western Blot test -35 days
3 Date of first positive HIV-1 p24 antigen/negative standard HIV-1 antibody enzyme immunoassay (EIA) -14 days
4 Patient clinical history information
5 Results of less sensitive HIV-1 EIA
n/a = not available

Table 3. List of MHC Class I Restricted Peptides Used as Stimuli

		Sequence	Restrictions
nef 1	(1-20)	GGKWSKSSVVGWPTVRERMR	B8
nef 7	(61-80)	EEEEVGFPVTPQVPLRPMTY	A1, B7, B35
nef 8	(71-90)	PQVPLRPMTYKAAVDLSHFL	A3 , A11
nef 9	(81-100)	KAAVDLSHFLKEKGGLEGLI	A11, B8, B40, Cw8
nef 10	(91-110)	KEKGGLEGLIHSQRRQDILD	A1, B60
nef 11	(101-120)	HSQRRQDILDEWIYHTQGYF	B7, B27
nef 12	(111-130)	LWIYHTQGYFPDWQNYTPGP	A1, B57
nef 13	(121-140)	PDWQNYTPGPGVRYPLTFGW	B7
nef 14	(131-150)	GVRYPLTFGWCYKLVPVEPD	A1, B18, B49
nef 18	(171-190)	GMDDPEREVEWRFDSRLAF	A25, A66
nef 19	(181-200)	EWRFDSRLAFHHVAREL	A1, A2, A3, A25, B52
nef 20	(191-210)	HHVARELHPEYFKNC	A1
RT 4	(18-26)	DGPKVKQWPLTEEKI	B8
RT 7	(32-47)	KALVEICTEMEKEGKI	A3
RT 9	(42-50)	EKEGKISKIGPENPYN	B51
RT 21	(103-117)	KKSVTVLDVGDAYGS	B35
RT 23	(113-127)	DAYFSVPLDEDFRKY	B51
RT 24	(118-132)	VPLDEDFRKYTAFTI	B35
RT 30	(148-162)	VLPQGWKGSPAIFQS	B51
RT 31	(153-167)	WKGSPAIFQSSMTKI	A3, A11, B7, B35
RT 35	(173-187)	KQNPDIVIYQYMDDL	B35
RT 36	(178-192)	IVIYQYMDDLYVGSD	A2
RT 38	(188-202)	YVGSDLEIGQHRTKI	A3
RT 41	(203-217)	EELRQHLLRWGLTTP	B44
RT 47	(233-247)	ELHPDKWTVQPIVLP	A2
RT 49	(243-257)	PIVLPEKDSWTVNDI	B57
RT 52	(260-271)	IQKLVGKLNWASQIYP	B15
RT 59	(293-307)	IPLTEEAELELAENY\R	B35
RT 62	(309-318)	EILKEPVHGVYYDPS	B15
RT 68	(338-352)	TYQIYQEPFKNLKTG	A11
RT 73	(363-377)	NDVKQLTEAVQKITT	A68
RT 75	(372-387)	VQKITTESIVIWGKTP	B57
RT 78	(388-402)	KFKLPIQKETWETWW	A32
RT 79	(393-407)	IQKETWETWWTEYWQ	B44
RT 86	(427-442)	YQLEKEPIVGAETFYV	B35
RT 87	(433-447)	PIVGAETFYVDGAAN	A68
RT 88	(438-452)	ETFYVDGAANRETKL	A66
RT 89	(443-457)	DGAANRETKLGKAGY	A29
RT 90	(448-462)	RETKLGKAGYVTNKG	B14
RT 99	(496-505)	VNIVTDSQYALGI IQ	B14
RT 106	(528-542)	KEKVYLAWVPAHKGI	B7
RT (476-484)	(476-484)	ILKEPVHGV	A2
p17.3	(21-35)	LRPGGKKKYKLKHIV	A3, A24, B8
p17.4	(31-45)	LKHIVWASRELERFA	B35
p17.8	(71-85)	GSEELRSLYNTVATL	A1, B8
p17.9	(81-95)	TVATLYCVHQRIDVK	A11
p17.13	(121-137)	AAGTGNSSQVSQNY	B35
p24.1	(1-20)	PIVQNLQGQMVHQAISPRTL	Cw3

p24.2	(11-30)	VHQAISPRTLNAWVKVVEEK	A2, A25, B7, B57
p24.3	(21-40)	NAWVKVVEEKAFSPEVIPMF	B57
p24.4	(31-50)	AFSPEVIPMFSALSEGATPQ	A66, Cw1
p24.5	(41-60)	SALSEGATPQDLNTMLNTVG	B7, B40, B60
p24.7	(61-80)	GHQAAMQMLKETINEEAAEW	B39, B52
p24.8	(71-90)	ETINEEAAEWDRVHPVHAGP	A25, A66
p24.11	(101-120)	GSDIAGTTSTLQEQIGWMTN	B57
p24.13	(121-140)	NPPIPVGEIYKRWIILGLNK	B8, B35
p24.14	(131-150)	KRWIILGLNKIVRMYSPTSI	B27, B62
p24.15	(141-160)	IVRMYSPTSILDIRQGPKEP	B52
p24.17	(161-180)	FRDYVDRFYKTLRAEQASQD	A24, A66, B14, B18, B44
p24.18	(171-190)	TLRAEQASQDVKNWMTETLL	B44, B57
p24.20	(191-210)	VQNANPDCKTILKALGPAAT	B8
p24.22	(211-230)	LEEMMTACQGVGGPGHKARV	A11
p17(77-85)	(77-85)	SLYNTVATL	A2
Env 1922	(586-598)	EKLWVTVYYGVPVWKEATTT	A3, A11, Cw7
Env 2023	(656-673)	ERYLKDQQLLGF	A24, B8, B14
Env 2043	(786-795)	IVELLGRRGWEVLKYWWNLL	B27
Env 2049	(843-851)	LHIPTRIRQGLERALL	B7
CMV	NB27	NLVPMVATV	A2
EBV	NB25	RPPIFIRRL	B7
EBV	NB26	FLRGRAYGL	B8

Table 4. Within Group Differences

		Group 1	Group 2	Group 2a	Group 2b	Group 3	Group 4	Group 1+ 2a	Group 2b + 3
Breadth	Baseline	60/76 (79%)	95/118 (81%)	29/41 (70%)	66/77 (86%)	46/73 (63%)	49/99 (49%)	89/117 (76%)	112/150 (75%)
	12 months	70/77 (92%)	66/118 (56%)	29/41 (70%)	37/77 (48%)	25/73 (34%)	36/99 (36%)	99/118 (84%)	62/150 (42%)
	p-valuea	0.3188	0.0479	1	0.0177	0.0277	0.1466	0.3437	0.0016
Magnitude	Baseline	3261 (131-3872)	4227 (0-12341)	1013 (132-4876)	9442 (0-10563)	6193 (777-9756)	1446 (260-2950)	2930 (131-4876)	7657 (0-10563)
	12 months	4674 (1001-6953)	1895 (0-6525)	2600 (422-6525)	1137 (0-2244)	2120 (53-4144)	596 (266-2392)	2972 (422-6953)	1215 (0-4144)
	p-valueb	0.5448	0.6622	0.7373	0.0693	0.7373	0.0951	0.3822	0.0289

[a] *Fisher P-exact test for baseline versus 12 months*
[b] *Wilcoxon matched pairs test for baseline versus 12 months*

Figure 1. Longitudinal assessment of HIV-specific effector responses to major histocompatibility complex (MHC) class I restricted panels of HIV peptides. Stacked bar graphs show changes with time in the number of spot forming cells per million peripheral blood mononuclear cells (SFC/10^6 PBMC) generated following stimulation of lymphocytes from HIV infected patients starting HAART at various times after infection with a panel of major histocompatibility complex (MHC) class I restricted HIV peptides. The x-axis indicated, for each bar, the number of days on highly active antiretroviral therapy (HAART) at the time tested. The legends list the HIV peptides tested for each study subject. Peptides are identified by the HIV gene product from which they were derived. Also shown are changes in $\log_{10}$ viral load keyed to the right hand y-axis. Results are presented for 1 subject in Group 1 (starting HAART before seroconversion, Panels A), 11 subjects in group 2 (starting HAART post seroconversion but in early HIV infection, Panels B to L), 1 subject in group 3 (starting HAART post seroconversion and in the second half of the first year of infection, Panel M) and 1 subject in group 4 (starting HAART in the chronic phase of infection, Panel N). The shaded area in Panels I, M and N correspond to changes in reactivity to Epstein-Barr virus derived control peptides with time.

Figure 2. Comparison of viral load, breadth and magnitude of responses between subjects in primary infection who maintain or lose HIV-specific effector activity following initiation of HAART. Subjects in PI were separated based on whether they maintained or lost effector responses specific for a panel of major histocompatibility complex restricted HIV peptides. Group 1+2a included 6 subjects staring HAART pre-

seroconversion (group1) and five recently infected persons who started HAART post-seroconversion (group 2a). Group 2b+3 included 6 recently infected individuals who started HAART post-seroconversion (group 2b) and 5 persons who began HAART in the second half of the first year of infection (group 3). Shown are scatter plots of viral load at baseline (panel A), breadth (percent of reactive peptides) of HIV-specific responses at baseline and on-therapy time point (panel B) and magnitude (number of spot forming cell per million peripheral blood mononuclear cells [SFC/10^6 PBMC]) of HIV-specific responses at baseline and on-therapy time point (panel C). The on-therapy data was generated from a lymphocyte sample taken at the closest available time point to 12 months on therapy as described in the results.

Figure 3. Longitudinal assessment of changes in the frequency of recently stimulated Interferon-γ (IFN-γ) producing HIV-1 Gag p55 specific CD4+ and CD8+ T cells. Panel A represents the control and p55 pool stimulated responses for subject MQPI006 at baseline. Bar graphs show the frequency (in percent) of CD69+ peripheral blood mononuclear cells producing IFN-γ in response to a pool of 122 15-mer peptides with 11 amino acid overlaps corresponding to HIV-1 Gag p55. Panel A displays a representative dot plot showing in the top right hand corner of each plot the percentage of CD69+ IFN-γ+ CD4+ generated following a 6-hour stimulation with no antigen (left) and the Gag p55 peptide pool (middle). Also shown is a dot plot of the CD69+ IFN-γ+ CD8+ cells generated following a 6-hour stimulation with the Gag p55 peptide pool (right). Longitudinal samples were analysed for HIV specific activity in the CD4+ (Panel B) and CD8+ T cell compartment (Panel C) from 4 subjects from group 1, 7 subjects from group

2, 2 subjects from group 3 and 2 subjects from group 4 were stimulated with no antigen and the Gag p55 peptide pool. The frequency of IFN-γ producing cells generated following stimulation with no peptide has been subtracted from the results shown. A frequency of 0.1% over background stimulation is considered a positive response.

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Chapter 4: Longitudinal Analysis of HIV-Specific Effector Responses in Untreated HIV PI

Data from chapter 3 demonstrates that HAART, the standard of care for HIV infection, plays an important role in shaping the antiviral immune response. Subjects initiating therapy early enough in infection to preserve HIV specific CD4+ T cell responses appear to maintain consistent HIV specific CD8+ T cell activity over the follow up period. Conversely, subjects who are treated at a time when HIV specific helper activity is not recovered, exhibit significant contractions in both the magnitude and the breadth of their HIV specific effector activity over time.

Removal of HIV antigen in the setting of chronic infection, and as mentioned in chapter 3 in patients later in the first year of infection, leads to the contraction of the immune response in subjects that have lost HIV specific T cell help. Thus changes in antigen level and within the antigen sequence may have a profound impact on the shape of the immune response, and thus it is clear that therapy interferes with the natural evolution of the host immune response.

A subset of subjects recruited in the first year of infection into the Quebec PI cohort, elect to remain untreated. Follow up of the changes that occur in the antiviral immune response pattern in these subjects offered a unique opportunity to study the natural evolution of these responses over time, reflecting the dynamic interplay between the host and virus at this early critical stage of disease.

ASSESSMENT OF LONGITUDINAL CHANGES IN HIV-SPECIFIC EFFECTOR ACTIVITY IN SUBJECTS UNDERGOING UNTREATED PRIMARY HIV INFECTION

Running Title: HIV-specific immunity in untreated primary HIV infection

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Abstract

Despite the failure of HIV-specific responses to clear the virus, they play a critical role in the control of viral replication throughout HIV infection. Untreated individuals, recruited in HIV primary infection (PI), were monitored for the natural evolution of HIV-specific immune responses starting in early HIV disease. Longitudinal analysis of changes in the magnitude and breadth of HIV specific responses to a panel of major histocompatibility complex class I restricted peptides was performed using the quantitative interferon-γ ELISPOT assay. Although immune responses were detected in all individuals at all times tested, the pattern of the immune responses differed significantly from that seen in subjects treated in early PI. Untreated PI subjects exhibited dramatic changes with time in the frequency of individual HIV peptide-specific CD8+ and CD4+ T cell responses whereas subjects who were treated early enough in infection to preserve HIV-specific activity did not. The overall magnitude of HIV-specific reactivity persisted over at least 12 months whereas the number of peptides recognized declined. Given that a significant relationship existed between the magnitude of the HIV specific response and viral load, it is likely that these effector cell expansions and contractions are driven by changes in antigen load.

INTRODUCTION

HIV-specific T cells are thought to play a central role in controlling HIV infection. The temporal association between the decline in peak viremia levels observed in acute infection and the appearance of HIV-specific CD8+ T cell responses[1,2] as well as increases in viral load with decline in these responses in late stage disease supports the importance of HIV-specific effector cells in suppressing HIV replication[3-5]. The strongest evidence of a role for HIV-specific CD8+ T cells in suppression of viral replication comes from animal models. In macaques infected with simian immunodeficiency virus (SIV) depletion of CD8+ T cells results in an increase in viral load [6,7]. CD8+ cytotoxic T lymphocytes (CTL) appear to exert an immune pressure on the virus sequences they recognize as demonstrated by the preferential emergence of viral escape mutations in sequences corresponding to CTL epitopes in both SIV-infected macaques and HIV-infected humans[8-14].

The pattern of the immune response induced during PI appears to determine viral set point and the subsequent course of infection[15-18]. Despite the development of HIV-specific immunity in HIV primary infection (PI), these responses do not clear the virus infection and disease progresses without treatment in most infected individuals. Highly active antiretroviral therapy (HAART) can suppress the replication of HIV in most anti-retroviral therapy naïve patients adhering to their drug regimens[19, 20]. Initiating HAART is recommended for acute HIV-1 infection to reduce viral dissemination and its harmful effects on the immune system[21-24].

While 69% of HIV infected subjects within the Quebec PI cohort reported having had symptoms of acute infection[25], not all cases of HIV infection are detected and

diagnosed during this acute stage. Many individuals whose infection is diagnosed after seroconversion may remain untreated for months to years. We have shown previously that starting treatment during acute infection/early disease (AIED) preserves HIV specific effector and helper activity in a significant proportion of subjects[21]. The beneficial effect on the maintenance of HIV-specific responses of starting HAART is lost sometime during early disease such that almost all individuals who initiate treatment 6 months or later after infection exhibit a decline in the breadth and magnitude of their HIV-specific CD4+ and CD8+ T cell responses in association with viral load control. These observations underline the importance of virus host interactions occurring in HIV PI on subsequent disease course. Characterization of the immune response to the virus at this time in infection is a step towards understanding the dynamic interaction between the virus and the host response and how this interaction impacts on disease course.

We present here the results of a longitudinal study on 12 subjects enrolled in the Quebec PI cohort who elected to remain untreated after HIV infection was diagnosed. We examined changes in the breadth and magnitude of CD8+ T cell effector responses to a panel of major histocompatibility complex (MHC) class I restricted peptides in these individuals. Using a quantitative HIV-peptide specific Interferon-γ (IFN-γ) enzyme linked immunospot (ELISPOT) assay, we show that untreated subjects exhibit expansions and contractions in the frequency of cells specific for individual peptides not seen in individuals at a similar stage of infection who start HAART. The overall magnitude of the responses to the peptide panels tested do not change significantly over the course of a 12 month period whereas the number of peptides recognized declines.

Methods:

Study population: In this report we present results generated in 17 subjects enrolled in the Quebec HIV PI cohort. Five of these subjects started HAART at various times during PI and are a subset of a larger group of individuals for whom information of HIV-specific immune responses have been previously reported[21] . The institutional review boards of all participating sites approved the study and all participants signed informed consent. All 17 subjects were HAART naïve at study entry. Subjects enrolled in this cohort were followed clinically at study entry and at week 2, 4, 6, 8 and every 3 months until month 24. We compared 12 subjects electing to remain untreated with 5 previously reported subjects who started HAART during PI. MQPI008 began therapy 99 days, MQPI009 102 days, MQPI010 47 days, MQPI011 53 days and MQPI013 195 days from infection. HAART consisted of at least two reverse transcriptase inhibitors and at least 1 protease inhibitor or non-nucleoside transcriptase inhibitor.

Entry criteria for the Quebec HIV PI cohort have been described previously[26]. The presumed date of infection was estimated for each individual using clinical and laboratory data as well as patient history information. The following guidelines proposed by the Acute HIV Infection and Early Disease Research Program sponsored by the National Institutes of Allergy and Infectious Disease Division of AIDS (Bethesda, MD) were used to estimate the date of infection: the date of the first indeterminate Western blot minus 35 days; the date of a positive HIV RNA test or p24 antigen assay available on the same day as a negative standard HIV enzyme immunoassay (EIA) test minus 14 days. The date of onset of symptoms of an acute retroviral syndrome minus 14 days was also used to estimate the date of infection. Information obtained from questionnaires

addressing the timing of high-risk behavior for HIV transmission was used when available to confirm the presumed date of infection. Study entry for each individual was defined as the date of the first clinic visit.

In this report we define acute infection as pre-seroconversion when viral load levels are usually at their peak 3 to 6 weeks after infection. A study subject is seropositive if their plasma samples produce a positive result in a standard HIV EIA and detect at least 3 bands in a confirmatory Western blot. Seroconversion occurs approximately 6 to 8 weeks from infection[27-32]. Early disease refers to the period after seroconversion when subjects are still negative in a less sensitive HIV-1 EIA (detuned assay). Plasma from individuals in early disease are therefore seropositive but generate an optical density result in the detuned EIA assay of less than 1.0 when they are within 170 days of infection (95% confidence intervals 163 to 183 days)[33,34].

Laboratory testing: HIV-1 EIA antibody testing was performed at three university hospital sites. The detuned assay was carried out at the University of California, San Francisco, CA using the Vironostica HIV-1 EIA (Organon-Tecnika, Boxtel, the Netherlands). Western blot analysis for HIV-1 antibodies and p24 antigen capture assays were performed at the Laboratoire de Santé Publique du Québec (Ste. Anne de Bellevue, QC, Canada). Plasma viremia was measured using the Roche Amplicor Assay (Roche Diagnostics, Mississauga, ON, Canada) with detection limit of 500 HIV-1 RNA copies/ml of plasma. Plasma samples falling below the detection limit of this assay were retested using the ultrasensitive method (Ultradirect, Roche) with a

detection limit of 50 copies/ml of plasma. T cell subset distribution was measured by flow cytometry.

Cells and peptides: PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) and frozen in 10% dimethyl sulfoxide (DMSO, Sigma, St Louis MO) in 90% fetal calf serum (FCS, Montreal Biotech Inc., Montreal, QC, Canada). Subjects were typed for MHC class I antigen expression by the amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles[35] (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI). Genomic DNA for molecular HLA-typing was prepared from either fresh blood or Epstein-Barr virus (EBV) transformed B cell lines using the QIAamp DNA blood kit (Qiagen Inc., Mississauga, ON).

Peptide Selection: The HIV epitopes used for stimulation were chosen from the National Institutes of Health (NIH) HIV molecular immunology database[36]. Peptides of 9-, 15-, or 20-aa in length containing these sequences were obtained from the Medical Research Council AIDS Reagent Project (Hertz, UK) and the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Lyophilized peptides were diluted to 1 mg/ml in Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY) containing 10% DMSO and stored at -70°C. They were used at a final concentration of 10 μM (20 to 40 ug/ml, depending on the peptide size).

ELISPOT assay for single cell IFN-γ release: IFN-γ secretion by virus-specific cells was quantified by ELISPOT assay as described[26]. Stimulating peptides were chosen on the basis of their having amino acid sequence binding motifs for the MHC class I alleles expressed by the person being tested. Stimulatory panels contained peptides restricted to multiple (2 to 5) MHC class I alleles per subject. Media, containing the equivalent amount of DMSO as present in peptide stimulation conditions, was used as a negative control. Anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) was used as a positive control stimulus. Cells were plated at two concentrations ($2x10^5$ cells/well and $5x10^4$ cells/well) for each peptide condition. The frequency of reactivity to anti-CD3 and the EBV/cytomegalovirus (CMV) peptide pool stimuli occurring in longitudinally collected samples was used to control for between-time point variability in cell responsiveness. Results are expressed as spot forming cells (SFC)/10^6 PBMC following subtraction of negative controls. Negative control stimulation produced less than 5 spots per well in greater than 90% of experiments. The average number of spots in the negative control wells was 3.13 ± 3.12. In experimental wells the signal was considered positive if at least 10 spots per well were present, the number of spots obtained was proportional to the number of cells plated and the number of spots per well that was at least 2-fold greater than the negative control wells. The identity of IFN- γ secreting cells as CD8+ was confirmed by the reduction of SFC numbers following depletion of CD8+ cells with magnetic beads (Dynal, Lake Success, NY).

Statistical Analysis: Paired student t-tests were employed to assess within group differences at study entry and after 1 year of follow up. Fisher P-exact tests were utilized

to test the significance of within- group differences in the breadth of HIV responses. Spearman correlation was used to examine if a relation existed between the immune response and a number of clinical parameters such as the relation between the magnitude or the breadth and the viral load, CD4 or CD8 T cell numbers. After normalizing individual peptide-specific responses by taking into consideration their proportional contribution to the sum of the magnitude of all positive responses for a given time point, the variance of individuals peptide specific responses was examined using a variance score measure. Variance scores assessed, for each time tested, how much the individual peptide-specific responses varied compared with the mean response at all times tested and was obtained by dividing the number of SFC/10^6PBMC generated to a peptide at a particular time point by the average number of SFC/10^6 PBMC for all times tested. χ^{2-} analysis was employed to assess whether expansions and contractions occurred more frequently in the untreated population than in the treated subjects who preserved HIV-specific responses. Immune responses that were 3 standard deviations greater or lower than the average standard deviation of all peptide specific responses in the treated patients were considered as perturbations. P-values of less then 0.05 were considered significant.

RESULTS

Study Population:

Twelve untreated subjects and 5 treated subjects who were recruited to the Quebec PI cohort in the fist year of infection but were studied. Viral loads, CD4, and CD8 T cell counts at study entry, presumed date from infection, route of transmission, HLA type, are shown in Table 1. Individuals were a median 38 years old (range 17 to 50) and exhibited a median $\log_{10}$ viral load of 4.64 (range 1.8 to 5.5) at study entry. Subjects manifested median CD4 counts of 461 cells/ml (range100 to 960) and CD8 counts of 709 cells/ml (range 325 to1338). Subjects entered the study at different times from infection at a median of 105 days from the presumed date of infection (range 30 to 263 days).

Immune Responses:

The quantitative IFN-γ ELISPOT assay was employed to monitor the HIV-specific immune response to a panel of HIV peptides selected based on their ability to bind to HLA alleles expressed by each subject. Figure 1 shows results for the longitudinal assessment of HIV-specific effector responses to a panel of MHC class I restricted peptides in three categories of Quebec PI cohort participants. Figure 1A displays data generated for one individual who is representative of 11 others tested and reported elsewhere who were treated early enough in AIED to preserve HIV-specific immune responses[21]. Figure 1B shows results for 1 subject who is representative of 11 others reported elsewhere who started HAART later in PI and who exhibited declines in the breadth and magnitude of their HIV-specific immunity in association with viral load control[21]. This pattern is similar to that seen when subjects start treatment in the chronic

phase of infection[37-39]. Similar analyses are presented in Figure 1C-F for 6 representative subjects recruited at different times from infection in PI who elected not to be treated. Individuals starting HAART early enough in AIED to preserve the breadth and magnitude of HIV-specific immune responses also maintained the hierarchy of these responses. This pattern contrasts with that seen in patients treated later in the first year of infection (Fig.1B) and in untreated subjects (Figure 1C-F). While untreated subjects undergoing PI had immune responses to between 5 and 15 HIV peptides tested during the follow up period, these individual peptide specific responses appeared to expand and contract over time suggesting that amplifications and contractions of cell populations able to secrete IFN-γ in response to individual HIV epitopes occurred during the period being studied.

To assess the extent of expansion and contraction in the hierarchy of the HIV-specific response observed in untreated individuals, in contrast to that seen in the other study groups, a variance score was generated that assigned a value to the degree of variation within individual peptide specific responses at each time tested. The variance score was calculated by dividing the number of SFC/10^6 PBMC generated to a peptide stimulus for a particular time point by the average number of SFC/10^6 PBMC generated to that stimulus at all times tested. To eliminate variation attributable to small changes in the cumulative magnitude of the response at each visit, individual peptide specific responses were normalized at each time with respect to the cumulative magnitude of that particular time point before the variance score was generated. Thus a score of 1 indicated no difference from the average reactivity to an individual peptide whereas a very large or small number indicated an increase or decrease in the number of peptide reactive cells at

that time compared to the average response. Longitudinal responses having a variance score approaching 1 at all times tested would indicate a stable hierarchy of peptide specific responses. The variance score pattern over time for 4 representative subjects that were treated early enough in PI to maintain the hierarchy of the response is represented in Figure 2A-D. All four patients appeared to have stable responses that fell within a narrow range around a variance score of 1. In contrast, the longitudinal delta score distribution of 4 subjects undergoing untreated PI showed that there were dramatic expansions (large delta scores) and contractions (low delta scores) in cells specific for individual peptides within this population. Moreover, expansions and contractions occurred significantly more frequently in the untreated than in the treated population ($p=0.01$, χ^2-test).

Individual peptide-specific responses were not present at all time points tested. Comparison of baseline versus 1-year follow up values for the breadth and the magnitude of HIV peptide specific immune responses revealed that there was a statistically significant decrease in the breadth of the response over the 1-year follow up, but not in the magnitude of the response (Figure 3A and B). The average magnitude of the response to the HIV peptide panels tested in these 12 untreated subjects was 3644 SFC/10^6 PBMC. After 1 year the average magnitude was 2887 SFC/10^6 PBMC (p=n.s., paired t-test). While 88% of all peptides tested were recognized at baseline only 48% were recognized after 1 year of follow up ($p<0.001$, χ^2-test).

Given that high viral loads have been correlated with the magnitude of HIV specific responses in chronically infected individuals, and that reduction of viral replication leads to dampening of effector activities as early as 3 months from infection[37-39], we questioned whether a relation existed between ongoing viral replication and both

the magnitude and the breadth of the immune response. Viral load was monitored at each time tested in order to assess its relationship to the breadth and magnitude of the immune response to the peptide panel tested. While no correlation existed between the breadth (Fig 4B) of the immune response and viral load, a statistically significant correlation was observed between the magnitude of the response to the peptide panel tested and viral load ($r=0.6$, $p<0.001$ spearman correlation)(Fig 4A).

Discussion:

We present here results describing patterns of change in the intensity and breadth of HIV-specific immune responses to a panel of MHC class I restricted peptides observed over a 1-year follow up in 12 subjects recruited during PI. Changes in the pattern of immune responses in treatment naïve subjects should reflect the natural evolution of the HIV-specific immune response. We have shown previously[21] that all subjects initiating HAART pre-seroconversion and 5 of 11 initiating treatment in early disease maintained both the magnitude and the breadth of their HIV specific immune responses (Fig1A). Concurrently, 6 of 11 subjects initiating therapy later in early disease and all subjects who started treatment beyond 6 months of infection experienced contractions in both of these parameters once therapy was initiated and viral load controlled[21] (Fig1B). In contrast, in subjects recruited during the first year of infection who remain treatment naïve, HIV peptide specific immune responses expand and contract such that the overall magnitude of the response to a stimulatory peptide panels used for screening persists while the number of peptide recognized falls. These expansions and contractions appeared to be governed by changes in viral burden, as a significant correlation existed between the magnitude but not the breadth of the immune response and changes in viral load.

Expansions and contractions of T cell receptor (TCR) variable beta (Vβ) chain usage in HIV PI have been described by others[40-43]. It was hypothesized that these changes in the TCR repertoire usage pattern was antigen driven and reflected shifts in HIV-specific effector activity. Our results confirm that this is the case by showing expansions and contractions in cells specific for HIV peptides. TCRVβ perturbations

appear to stabilize with initiation of HAART in PI likely due to reduction of antigen with therapy[42]. In this report, we show that significant perturbations are occurring in antigen specific T cell secretion of IFN-γ. Additionally, given the relationship between the magnitude of HIV specific activity and viral load, it appears that changes in viral replication, and therefore in antigen load, may have profound effects on the number of HIV peptide specific effector cells as it does on TCRVβ expression patterns.

Two potential immune mechanisms may account for changes in the immune response to viral infection during the course of untreated PI. Persistent viral replication and high doses of cognate antigen may lead to T-cell responses becoming terminally differentiated or deleted due to activation induced cell death[44]. Clonal exhaustion has been implicated in the loss of virus specific activity in a number of viral infections such as in Lymphocytic Choriomeningitis Virus (LCMV) and EBV infection[45]. In HIV infection data presented here show that virus specific effector functions change and shift over time in untreated early PI. Yet unlike LCMV infection, although HIV specific immune responses may not appear at all time-points, reemergence of specificities sometimes occurs later in follow up. This phenomenon suggests that although terminal differentiation may eliminate high affinity antigen-specific T cells, the global antiviral response appears to remain unaffected allowing for the reemergence of specificities during untreated infection.

Several studies have shown that virus from acutely infected individuals was homogeneous even when the infecting partner's virus was genetically diverse [46-48], probably reflecting the transmission of one or few viral isolates and/or replication of a dominant strain. Variation in the viral sequence becomes apparent as early as a few

weeks post infection, within the seronegative window[49,50]. This early heterogeneity of HIV most probably reflects the emergence of viral variants due to the host immune response[51]. Given that significant changes may occur in the sequence of CTL epitopes, HIV-specific cells that no longer recognize their cognate antigen may contract into a memory phase. As viral quasispecies are never lost completely, and are archived within established reservoirs, these variants may remerge during subsequent infection and reactivate these memory responses. Thus through constant competition for fitness between virus subspecies, antigen induced immune responses may reappear at later times from infection, and thus never be lost completely but may be clonally expanded and narrowed during the course of viral infection. It would be necessary to monitor virus specific immune responses to viral peptides derived from the sequence of the dominant autologous strain for each time tested in each individual to effectively address this issue.

In summary, the strength of the immune response to virus remains constant in the early phase of untreated HIV infection, while the breadth of the immune response contracts. While treatment early in infection has the potential to preserve the hierarchy of the immune response, the pattern of immunodominance shifts significantly in untreated HIV infection. It is plausible that antigen driven expansions and contractions of the immune response occurring during untreated PI may account for the erratic pattern of HIV peptide-specific immune responses. Characterization of responses that are integral to control of viral replication, or peptide-specific responses that are lost when viral load spikes, during this early period of intense immune activation may identify epitopes that are desirable targets for vaccine design. Additionally, monitoring the pattern of changes that occur in the viral sequence that account for this shifting pattern in the immune

response may contribute to a better understanding of the correlates of immunity that are important in establishing rates of disease progression.

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Figure 1

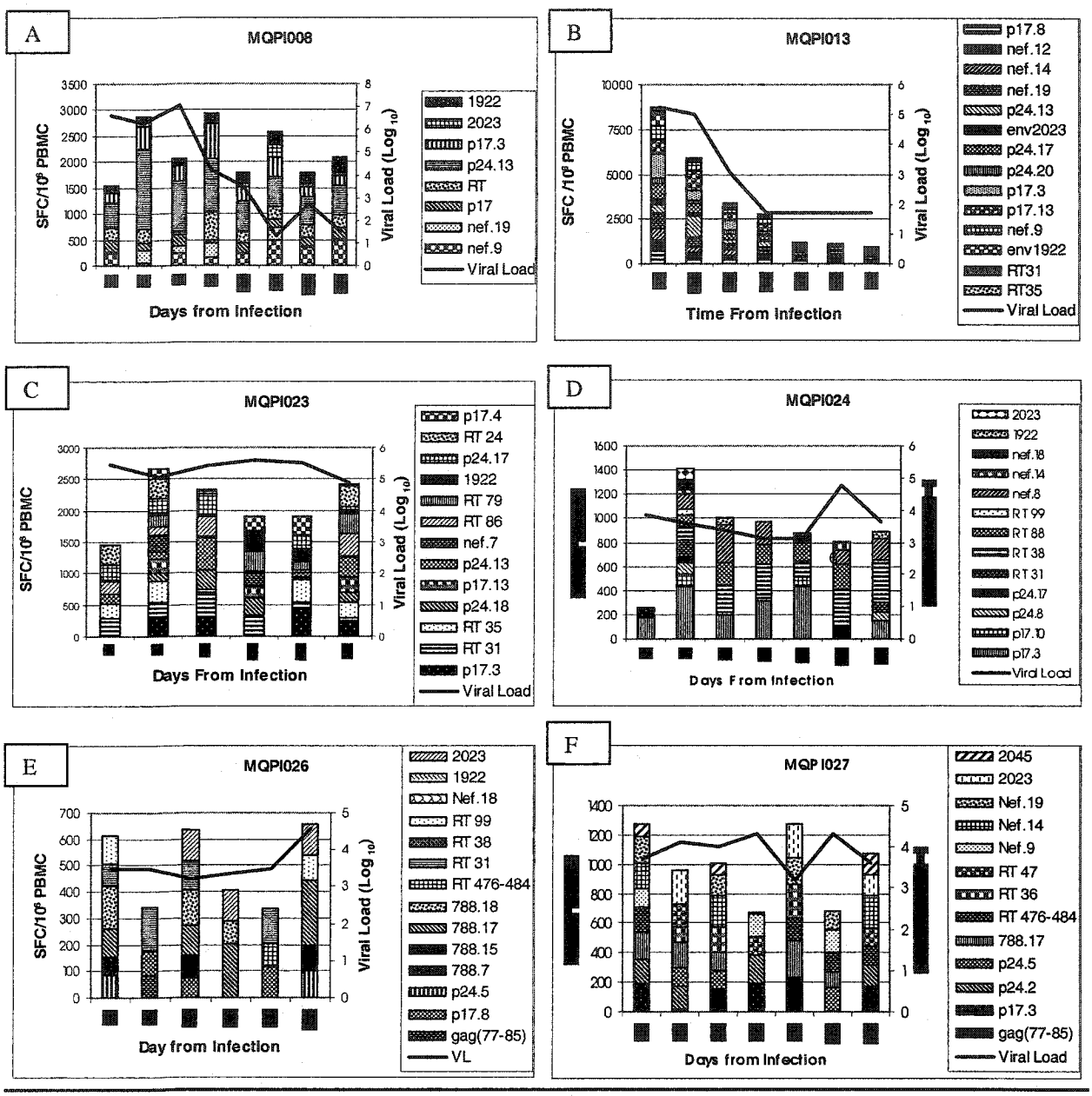

A
MQPI008
SFC/10⁶ PBMC
Viral Load (Log 10)
Days from Infection
1922
2023
p17.3
p24.13
RT
p17
nef.19
nef.9
Viral Load
B
MQPI013
SFC /10⁶ PBMC
Viral Load (Log 10)
Time From Infection
p17.8
nef.12
nef.14
nef.19
p24.13
env2023
p24.17
p24.20
p17.3
p17.13
nef.9
env1922
RT31
RT35
Viral Load
C
MQPI023
SFC/10⁶ PBMC
Viral Load (Log 10)
Days From Infection
p17.4
RT 24
p24.17
1922
RT 79
RT 86
nef.7
p24.13
p17.13
p24.18
RT 35
RT 31
p17.3
Viral Load
D
MQPI024
Days From Infection
2023
1922
nef.18
nef.14
nef.8
RT 99
RT 88
RT 38
RT 31
p24.17
p24.8
p17.10
p17.3
Viral Load
E
MQPI026
SFC/10⁶ PBMC
Viral Load (Log 10)
Day from Infection
2023
1922
Nef.18
RT 99
RT 38
RT 31
RT 476-484
788.18
788.17
788.15
788.7
p24.5
p17.8
gag(77-85)
VL
F
MQP I027
Days from Infection
2045
2023
Nef.19
Nef.14
Nef.9
RT 47
RT 36
RT 476-484
788.17
p24.5
p24.2
p17.3
gag(77-85)
Viral Load

Figure 2

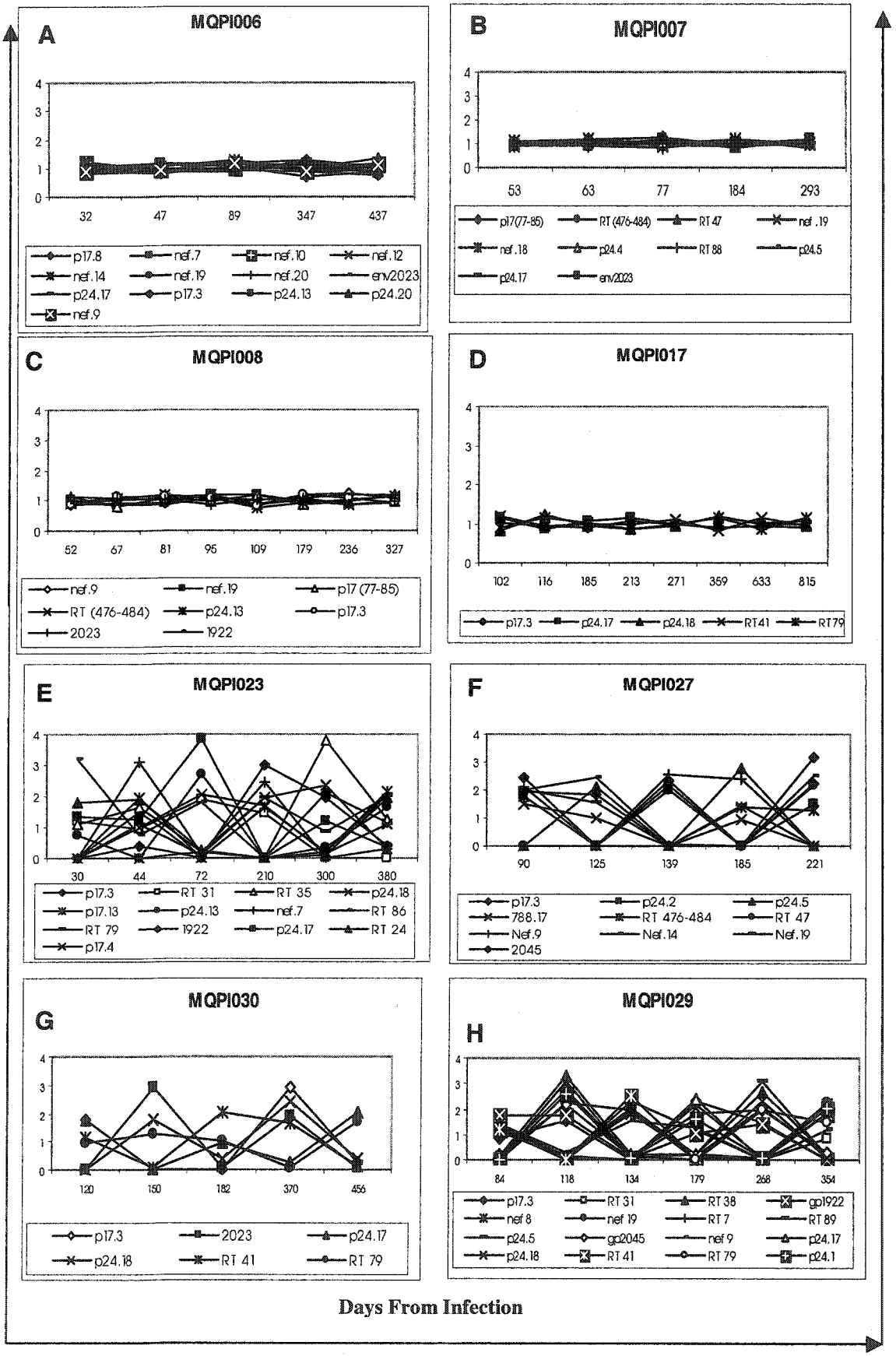
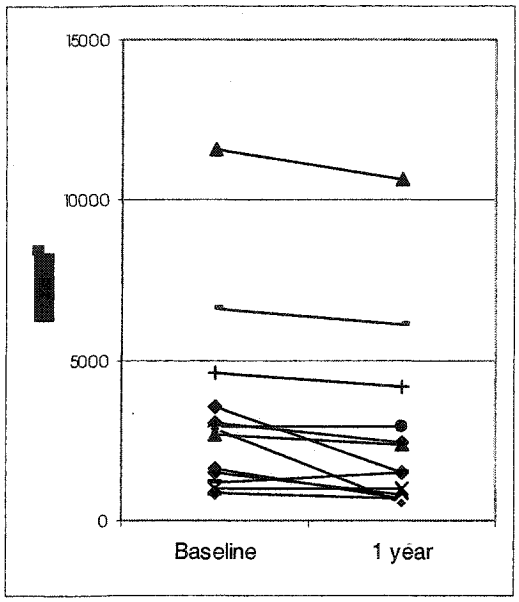

A
MQPI006
p17.8
nef.7
nef.10
nef.12
nef.14
nef.19
nef.20
env2023
p24.17
p17.3
p24.13
p24.20
nef.9
B
MQPI007
p17(77-85)
RT (476-484)
RT 47
nef.19
nef.18
p24.4
RT 88
p24.5
p24.17
env2023
C
MQPI008
nef.9
nef.19
p17 (77-85)
RT (476-484)
p24.13
p17.3
2023
1922
D
MQPI017
p17.3
p24.17
p24.18
RT41
RT79
E
MQPI023
p17.3
RT 31
RT 35
p24.18
p17.13
p24.13
nef.7
RT 86
RT 79
1922
p24.17
RT 24
p17.4
F
MQPI027
p17.3
p24.2
p24.5
788.17
RT 476-484
RT 47
Nef.9
Nef.14
Nef.19
2045
G
MQPI030
p17.3
2023
p24.17
p24.18
RT 41
RT 79
H
MQPI029
p17.3
RT 31
RT 38
gp1922
nef 8
nef 19
RT 7
RT 89
p24.5
gp2045
nef 9
p24.17
p24.18
RT 41
RT 79
p24.1
Spot Forming Cells
Viral Load
(Log10)
Days From Infection

Figure 3

A


15000
10000
5000
0
Baseline
1 year

B

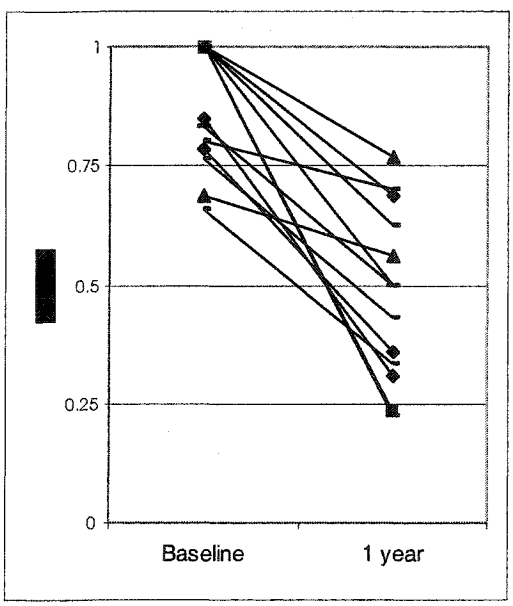

1
0.75
0.5
0.25
0
Baseline
1 year

Figure 4

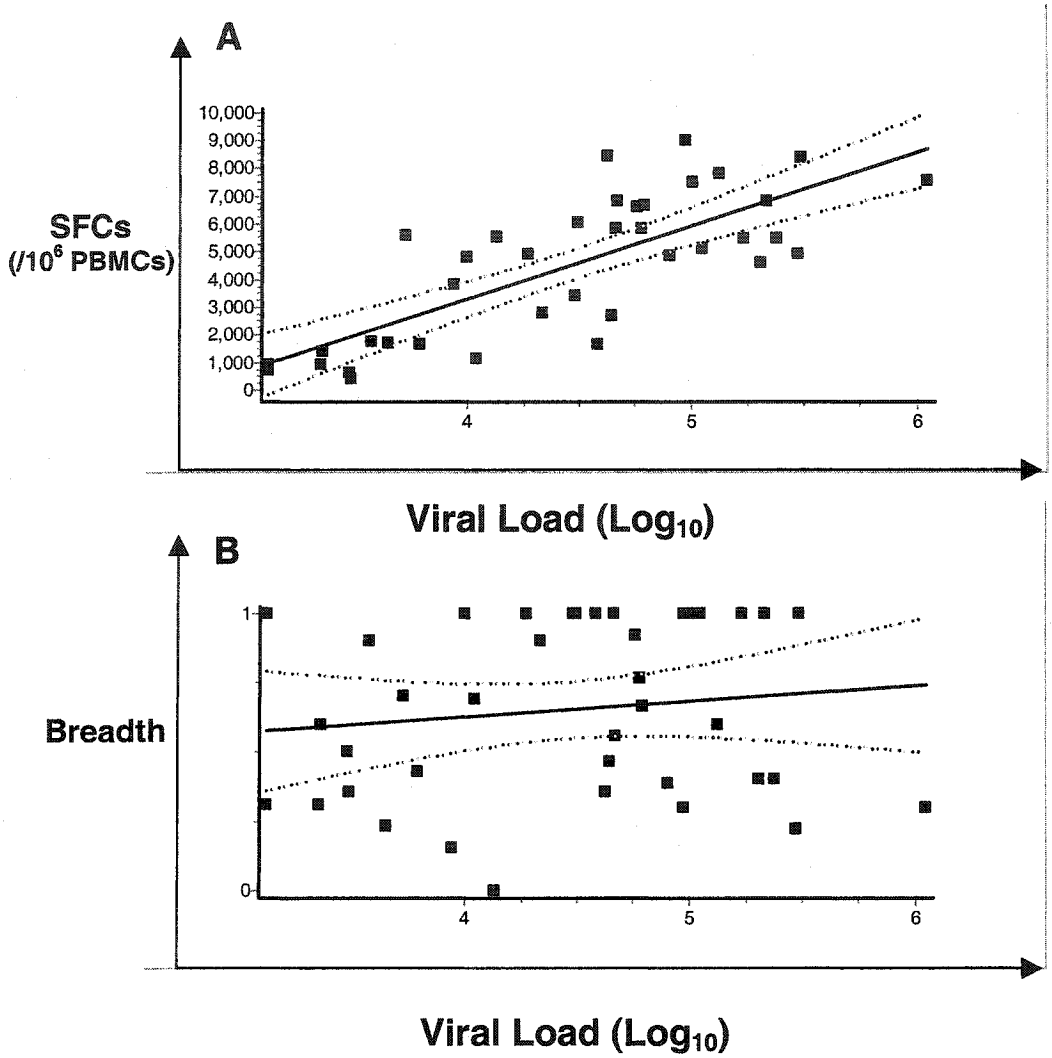

A
SFCs
(/10^6 PBMCs)
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
4
5
6
Viral Load (Log10)
B
Breadth
1
0
4
5
6
Viral Load (Log10)

Table 1. Population Characteristics

	Days from Infection	Viral Load	CD8	CD4	Age	Sex	Route of Transmission	Symptoms	A Locus		B Locus		C Locus		Treatment
MQPI006	32	3.34	720	330	36	M	MSM	yes	A1	A24	B8	B18	Cw5	Cw7	AZT, 3TC, Indinavir
MQPI007	53	3.1	1276	552	42	M	MSM	yes	A2	A25	B14	B56	Cw1	Cw3	d4T, 3TC, Indinavir
MQPI008	52	6.7	810	560	53	M	MSM	no	A1	A2	B8		Cw7		AZT, 3TC, Indinavir
MQPI013	76	5.25	2672	781	32	M	MSM	yes	A1	A24	B8	B35	Cw4	Cw7	AZT, 3TC, Indinavir
MQPI017	138	3.23	840	640	41	M	MSM	yes	A23	A31	B44		Cw4	Cw5	d4T, 3TC, Indinavir, Efavirenz
MQPI023	68	5.45	653	467	43	M	hetero	no	A3	A23	B35	B44	Cw4		n/a*
MQPI024	41	5.5	770	430	17	F	IDU	yes	A3	A66	B18	B41	Cw7	Cw17	n/a*
MQPI025	60	4.83	555	333	33	M	MSM	yes	A11	A24	B8	B35	Cw4	Cw7	n/a*
MQPI026	114	3.351	657	542	41	M	MSM	yes	A2	A3	B14	B52	Cw12	Cw8	n/a*
MQPI027	127	4.45	1638	822	26	M	MSM	yes	A2	A24	B18	B40	Cw3	Cw12	n/a*
MQPI028	90	1.8	736	432	37	M	MSM	yes	A2		B8	B57	Cw6	Cw7	n/a*
MQPI029	84	2.67	593	399	28	M	MSM	yes	A3	A2	B40	B44	Cw3	Cw16	n/a*
MQPI030	55	3.73	1540	960	39	M	MSM	yes	A23	A24	B44		Cw4	Cw5	n/a*
MQPI031	150	3.727	681	500	39	M	IDU	yes	A2	A24	B27	B40	Cw1	Cw3	n/a*
MQPI032	150	5.31	990	810	43	M	IDU	yes	A2	A3	B40	B49	Cw3	Cw7	n/a*
MQPI033	210	5.47	325	180	50	M	IDU	no	A31	A68	B40	B44	Cw2	Cw3	n/a*
MQPI034	263	5.5	1310	100	36	M	MSM	no	A3		B35	B15	Cw2	Cw3	n/a*

**n/a= not applicable*

Table 2. List of MHC Class I Restricted Peptides Used as Stimuli

Peptide ID (Sequence Location)		Sequence	MHC Restriction(s)
nef 1	(1-20)	GGKWSKSSVVGWPTVRERMR	B8
nef 7	(61-80)	EEEEVGFPVTPQVPLRPMTY	A1, B7, B35
nef 8	(71-90)	PQVPLRPMTYKAAVDLSHFL	A3 , A11
nef 9	(81-100)	KAAVDLSHFLKEKGGLEGLI	A11, B8, B40, Cw8
nef 10	(91-110)	KEKGGLEGLIHSQRRQDILD	A1, B60
nef 11	(101-120)	HSQRRQDILDEWIYHTQGYF	B7, B27
nef 12	(111-130)	LWIYHTQGYFPDWQNYTPGP	A1, B57
nef 13	(121-140)	PDWQNYTPGPGVRYPLTFGW	B7
nef 14	(131-150)	GVRYPLTFGWCYKLVPVEPD	A1, B18, B49
nef 18	(171-190)	GMDDPEREVEWRFDSRLAF	A25, A66
nef 19	(181-200)	EWRFDSRLAFHHVAREL	A1, A2, A3, A25, B52
nef 20	(191-210)	HHVARELHPEYFKNC	A1
RT 4	(18-26)	DGPKVKQWPLTEEKI	B8
RT 7	(32-47)	KALVEICTEMEKEGKI	A3
RT 9	(42-50)	EKEGKISKIGPENPYN	B51
RT 21	(103-117)	KKSVTVLDVGDAYGS	B35
RT 23	(113-127)	DAYFSVPLDEDFRKY	B51
RT 24	(118-132)	VPLDEDFRKYTAFTI	B35
RT 30	(148-162)	VLPQGWKGSPAIFQS	B51
RT 31	(153-167)	WKGSPAIFQSSMTKI	A3, A11, B7, B35
RT 35	(173-187)	KQNPDIVIYQYMDDL	B35
RT 36	(178-192)	IVIYQYMDDLYVGSD	A2
RT 38	(188-202)	YVGSDLEIGQHRTKI	A3
RT 41	(203-217)	EELRQHLLRWGLTTP	B44
RT 47	(233-247)	ELHPDKWTVQPIVLP	A2
RT 49	(243-257)	PIVLPEKDSWTVNDI	B57
RT 52	(260-271)	IQKLVGKLNWASQIYP	B15
RT 59	(293-307)	IPLTEEAELELAENY\R	B35
RT 62	(309-318)	EILKEPVHGVYYDPS	B15
RT 68	(338-352)	TYQIYQEPFKNLKTG	A11
RT 73	(363-377)	NDVKQLTEAVQKITT	A68
RT 75	(372-387)	VQKITTESIVIWGKTP	B57
RT 78	(388-402)	KFKLPIQKETWETWW	A32
RT 79	(393-407)	IQKETWETWWTEYWQ	B44
RT 86	(427-442)	YQLEKEPIVGAETFYV	B35
RT 87	(433-447)	PIVGAETFYVDGAAN	A68
RT 88	(438-452)	ETFYVDGAANRETKL	A66
RT 89	(443-457)	DGAANRETKLGKAGY	A29
RT 90	(448-462)	RETKLGKAGYVTNKG	B14
RT 99	(496-505)	VNIVTDSQYALGI IQ	B14
RT 106	(528-542)	KEKVYLAWVPAHKGI	B7
RT (476-484)	(476-484)	ILKEPVHGV	A2
p17.3	(21-35)	LRPGGKKKYKLKHIV	A3, A24, B8
p17.4	(31-45)	LKHIVWASRELERFA	B35
p17.8	(71-85)	GSEELRSLYNTVATL	A1, B8
p17.9	(81-95)	TVATLYCVHQRIDVK	A11
p17.13	(121-137)	AAGTGNSSQVSQNY	B35

p24.1	(1-20)	PIVQNLQGQMVHQAISPRTL	Cw3
p24.2	(11-30)	VHQAISPRTLNAWVKVVEEK	A2, A25, B7, B57
p24.3	(21-40)	NAWVKVVEEKAFSPEVIPMF	B57
p24.4	(31-50)	AFSPEVIPMFSALSEGATPQ	A66, Cw1
p24.5	(41-60)	SALSEGATPQDLNTMLNTVG	B7, B40, B60
p24.7	(61-80)	GHQAAMQMLKETINEEAAEW	B39, B52
p24.8	(71-90)	ETINEEAAEWDRVHPVHAGP	A25, A66
p24.11	(101-120)	GSDIAGTTSTLQEQIGWMTN	B57
p24.13	(121-140)	NPPIPVGEIYKRWIILGLNK	B8, B35
p24.14	(131-150)	KRWIILGLNKIVRMYSPTSI	B27, B62
p24.15	(141-160)	IVRMYSPTSILDIRQGPKEP	B52
p24.17	(161-180)	FRDYVDRFYKTLRAEQASQD	A24, A66, B14, B18, B44
p24.18	(171-190)	TLRAEQASQDVKNWMTETLL	B44, B57
p24.20	(191-210)	VQNANPDCKTILKALGPAAT	B8
p24.22	(211-230)	LEEMMTACQGVGGPGHKARV	A11
p17(77-85)	(77-85)	SLYNTVATL	A2
Env 1922	(586-598)	EKLWVTVYYGVPVWKEATTT	A3, A11, Cw7
Env 2023	(656-673)	ERYLKDQQLLGF	A24, B8, B14
Env 2043	(786-795)	IVELLGRRGWEVLKYWWNLL	B27
Env 2049	(843-851)	LHIPTRIRQGLERALL	B7
CMV	NB27	NLVPMVATV	A2
EBV	NB25	RPPIFIRRL	B7
EBV	NB26	FLRGRAYGL	B8

Figure 1. **Longitudinal assessment of HIV-specific effector responses to MHC class I-restricted panels of HIV peptides.** Stacked bar graphs show changes with time in the number of spot forming cells per million peripheral blood mononuclear cells (SFC/10^6 PBMC) generated after stimulating PBMC from HIV-infected patients with a panel of major histocompatibility complex (MHC) class I restricted peptides. The *x*-axis indicates, for each bar, the number of days elapsed from baseline (the first clinic visit). The legend at the right of each panel lists the HIV peptides tested for each study subject. Peptides are identified by the HIV gene product from which they were derived. For the location and amino acid sequence of each peptide see Table 2. Also shown are changes in the $\log_{10}$ viral load keyed to the right hand y-axis. Results are presented for one subject treated early in primary infection (PI) who maintained HIV specific CD4+ and CD8+ T cell responses after initiation of highly active antiretroviral therapy (HAART) (Panel A), one subject treated later in primary infection who lost HIV specific CD4+ and CD8+ T cell responses after initiation of HAART (Panel B), and 4 subjects recruited PI who remained untreated (Panel C-F).

Figure 2. **Longitudinal assessment of expansions and contractions in the HIV specific effector response.** Bar graphs display variance scores for individual HIV peptides tested at the follow up times indicated on the x-axis. The *y*-axis indicates the variance score (peptide specific response/mean peptide specific response for all times tested) for each peptide tested. The legend below each panel lists the HIV peptides tested for each study subject. Peptides are identified by the HIV gene product from which they were derived. For the location and amino acid sequence of each peptide see Table 2.

Results are presented for 4 subjects treated early in HIV primary infection who maintained HIV specific effector responses over time (Panel A-D) and 4 treatment naïve subjects (Panel E-H).

Figure 3. **Comparison of the cumulative magnitude and breadth of the HIV-specific effector response at baseline and 1 year of follow-up**. Shown are scatter plots illustrating the magnitude (number of spot forming cells per million peripheral blood mononuclear cells [SFC/10^6 PBMC]) (Panel A) and the breadth (number of peptides recognized/number of peptides tested) (Panel B) at baseline and 1 year of follow up for all untreated subjects studied.

Figure 4. **Analysis of the relation between the magnitude or breadth of the HIV specific responses and time**. Shown are scatter plots of the correlation analysis of magnitude (number of spot forming cells per million peripheral blood mononuclear cells [SFC/10^6 PBMC]) (Panel A) or the breadth (number of peptides recognized/number of peptides tested) (Panel B) over the follow-up period for all untreated subjects.

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Chapter 5: Evaluation of the Matrix ELISPOT

Peptide panel ELISPOTs are commonly employed to monitor immune responses to HIV (chapter 2, 3 and 4). The great limitation to this technique is that the panels are assembled based on epitopes characterized in patients in chronic infection. Given that the immune response appears to evolve with time leading to striking differences in the response patterns in early versus later stages of infection (chapter 2), the use of peptide panels may overlook a great deal of previously uncharacterized responses. Thus chapter 5 focuses on a novel strategy, that may overcome the limitations of the peptide panel ELISPOT, for screening immune responses across all expressed HIV gene products in an unbiased manner.

EVALUATION OF THE MATRIX ELISPOT

Running Title: Mapping the HIV-specific effector response

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Key Words: Human, CTL, T-Lymphocytes, peptides

Manuscript in preparation

5.1 Absract:

New approaches combining peptide sets spanning all expressed HIV genes and the high throughput enzyme linked immunospot (ELISPOT) assay should provide a more complete picture of the number and location of peptides recognized as well as of the frequency of cells recognizing these peptides in HIV infected persons than was previously possible. Here we have assessed the utility and the applicability of peptide pool-matrices to screen peripheral blood mononuclear cells (PBMC) from 2 HIV infected subjects for HIV-specific immune responses using an ELISPOT assay. This approach assessed the breadth, magnitude and specificity of PBMC secreting IFN-γ in response to peptide stimuli derived from all expressed HIV genes. The matrix with verification approach improved our ability to describe these parameters for cells that secrete IFN-γ in response to HIV peptides. The approach taken here for assessing HIV-specific responses overcomes limitations inherent in selecting peptide panels as stimuli to describe the frequency and specificity of HIV-specific cells in HIV infected individuals. This type of analysis is a first step towards identifying the regions of the virus that may be important for control of viral replication at different phases of HIV disease and may be useful for identifying the correlates of protection against progression.

5.2 Introduction:

Several lines of evidence support a role for cytotoxic T lymphocytes (CTL) in the control of viremia in human immunodeficiency virus (HIV) infection. In acute infection, CTL induction is temporally associated with a reduction in plasma viremia[9,20]. In late stage disease, as CTL function declines, a rise in viremia occurs[19,24]. Selection for mutations within sequences recognized by CTL provides evidence that these immune responses exert pressure that suppresses viral replication[4,15,18,21,22,25]. The most direct support for virus specific CTL suppressing viral replication comes from studies in an animal model for HIV infection, i.e. macaques infected with simian immunodeficiency virus (SIV). CD8+ T cell depletion in macaques results in uncontrolled viremia and rapid disease progression[16,26].

Previous work aiming to describe the HIV specific immune response have relied on the use of peptide panels based on epitopes recognized mainly by chronically infected individuals restricted by particular HLA alleles[5,6,8,13,23]. This approach was chosen because it balanced obtaining a picture (albeit limited) of the magnitude and breadth of the HIV-specific immune response and how this changes with time and a variety of interventions with the high cost of peptides sets spanning all expressed HIV genes and limitations in the availability of cells at any particular time point. There are two main drawbacks to using such selected peptide panels to assess the pattern of HIV-specific immune responses of subjects infected with HIV. Firstly, peptides that are selected because they are immunodominant in a population in the chronic phase of infection may not be the same as those that are immunodominant in subjects at other stages of infection, who spontaneously control viremia such as long-term non progressors or who remain

uninfected despite exposure to HIV. A number of reports provide evidence that this is in fact the case[6,8,12,14,17]. The second limitation lies in the fact that testing a set number of peptides may overlook the true breadth and magnitude of the HIV-specific response as not all epitopes presented by any particular HLA allele have been described[3,12]. In order to more fully describe HIV specific immune responses, it would be necessary to test peptides spanning all gene products.

Availability of HIV peptide sets corresponding to all expressed HIV genes and high throughput assays such as the enzyme linked immunospot (ELISPOT) assay have permitted the design of screening strategies for HIV-specific immune responses that are unbiased with respect to previously described approaches based on major histocompatibility complex (MHC) class I restriction specificity[3,12]. Using such approaches should provide a more complete picture of the number and location of peptides recognized as well as of the frequency of cells recognizing these peptides in HIV infected persons than was previously possible. This type of analysis is a first step towards identifying the regions of the virus that may be important for control of viral replication at different phases of HIV disease and may be useful for identifying the correlates of protection against progression.

In this report, we have assessed the utility and the applicability of peptide pool-matrices to screen peripheral blood mononuclear cells (PBMC) from 2 HIV infected subjects for HIV-specific immune responses using an ELISPOT assay. This approach assessed the breadth, magnitude and specificity of PBMC secreting IFN-γ in response to peptide stimuli derived from all expressed HIV genes.

5.3 Methods:

Study subjects: Two HIV infected subjects were analyzed. The institutional review board of the study site approved this work and the participants signed informed consent. The presumed date of infection was estimated for each individual using clinical and laboratory data as well as patient history information. The following guidelines proposed by the Acute HIV Infection and Early Disease Research Program sponsored by the National Institutes of Allergy and Infectious Disease Division of AIDS (Bethesda, MD) were used to estimate the date of infection: The date of the first indeterminate Western blot minus 35 days; The date of a positive HIV RNA test or p24 antigen assay available on the same day as a negative standard HIV enzyme immunoassay (EIA) test minus 14 days. The date of onset of symptoms of an acute retroviral syndrome minus 14 days was also used to estimate the date of infection. Information obtained from questionnaires addressing the timing of high-risk behavior for HIV transmission was used when available to confirm the presumed date of infection.

Laboratory testing: HIV-1 EIA antibody testing was performed at three university hospital sites. The detuned assay was carried out at the University of California, San Francisco, CA using the Vironostica HIV-1 EIA (Organon-Tecnika, Boxtel, the Netherlands). Western blot analysis for HIV-1 antibodies and p24 antigen capture assays were performed at the Laboratoire de Santé Publique du Québec (Ste. Anne de Bellevue, QC, Canada). Plasma viremia was measured using the Roche Amplicor Assay (Roche Diagnostics, Mississauga, ON, Canada) with detection limit of 500 HIV-1 RNA copies/ml of plasma. Plasma samples falling below the detection limit

of this assay were retested using the ultrasensitive method (Ultradirect, Roche) with a detection limit of 50 copies/ml of plasma. T cell subset distribution was measured by flow cytometric analysis.

Cells: PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) and frozen in 10% dimethyl sulfoxide (DMSO, Sigma, St Louis MO) in 90% fetal calf serum (FCS, Montreal Biotech Inc., Montreal, QC, Canada).

HLA typing: Subjects were typed for MHC class I antigen expression by the amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles[11] (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI). Genomic DNA for molecular HLA-typing was prepared from Epstein-Barr virus transformed B cell lines using the QIAamp DNA blood kit (Qiagen Inc., Mississauga, ON).

Peptide Panel Selection: Results generated using The HIV peptide panels as stimuli were compared to results generated using the HIV peptide pool matrix approach. HIV peptide panels were selected from the National Institutes of Health (NIH) HIV molecular immunology database based on their predicted ability to bind the HLA alleles of the subjects being tested[10].

Design of Peptide Matrices: The HIV peptide sets used for stimulation were either 15 amino acid (aa) with 11 aa overlaps (Gag, Env, Nef, Tat, Rev, Vpr, Vpu, Vif) or 20 aa

with 10 aa overlaps (Pol). The peptides were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Lyophilized peptides (n=621) spanning all HIV-1 gene products were dissolved to a final concentration 10 mg/ml in DMSO (Sigma) and stored at -70°C. These included 100 Pol 20- mers and 123 Gag 15 mers corresponding to the HIV-1_{HXB2R} clade B isolate , 49 Nef, 27 Rev, 23 Tat, 46 Vif, 22 Vpr and 19 Vpu 15-mers corresponding to consensus HIV ([Vpu] or consensus clade B sequence (Nef, Rev, Tat, Vif, Vpr) and 212 Env-15 mers corresponding the HIV_{MN} clade B isolate. Pools containing 2-15 peptides were prepared and organized into matrices of Gag, Pol, Nef, Env and accessory gene peptide pools such that each peptide was present in two pools within each peptide matrix.

ELISPOT assay for single cell IFN-γ release: IFN-γ secretion by HIV-specific cells was quantified using the ELISPOT assay. Cells were plated at 6 x 10^4 to 10^5 PBMCs per well and were stimulated in round-bottomed plates with the peptide pools for 3 hours. The final concentration of each peptide within a pool was 2 to 4 μg/ml. Media alone was used as a negative control and anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) was used as a positive control stimulus. Results are expressed as spot forming cells per million peripheral blood mononuclear cells (SFC/10^6 PBMC) following subtraction of negative controls. Negative control stimulation produced less than 5 spots per well in greater than 90% of experiments. The average number of spots in the negative control wells was 3.13 ± 3.12. In experimental wells the signal was considered positive if at least 10 spots per well were present and the number of spots was at least 3-fold greater than the negative control wells.

Confirmation of Peptide Specificity: The stimulatory capacity of candidate stimulatory peptides identified in the peptide pool matrix ELISPOT assay was confirmed in a second experiment with cells from the same time point stimulated with individual peptides that were common to two stimulatory peptide pools in the initial ELISPOT screen. Verification peptide panels consisted of 4-64 potential peptide candidates. For peptide verification experiments, cells were plated in triplicates with 4 μg/ml of individual candidate peptides. Responses were considered positive if the number of spots per well were at least 2 fold over background and over 50 SFC/10^6 cells.

5.4 Results:

HIV Specific Effector Responses: HIV peptide pool matrices corresponding to peptides spanning all expressed HIV genes were employed to assess the feasibility and advantages of the matrix strategy compared with the previously used stimulatory peptide panel experiments in PBMCs from the 2 HIV infected subjects. Specificities identified in the peptide poll matrix strategy were then compared to the peptides that would have been selected for testing based on the peptide panel strategy.

HTM 305 is a male who was infected at 39 years of age on the 15th of March of 1996. HTM 305 was treated with a 10-day cycle of AZT and 3TC in June of 1996, and elected to remain untreated for the remainder of his follow up. HTM 305 had the following HLA type: A11, A24, B8, B35, Cw4, Cw7. PBMC from May 15th, 2002 were used for the experiments described here, at a time when the subject was untreated and in the chronic phase of infection. At the time tested his CD4 count was 222 cells/μl and he had a viral load of <50 copies/ml. HTM 310 is a female who was infected at 22 years of age on the presumed date of February 20th, 1997 via heterosexual contact. HTM 310 started treatment on April 1st, 1997 with D4T/3TC/Ritonavir, and 5 months later began Indinavir to replace previously prescribed Ritonavir. HTM 310 had the following HLA types: A11, A68, B27, B57, Cw1, Cw6. PBMC from June 19th, 2002 were used for the experiments described here. At this time point HTM 310 had been on successful HAART continuously since April 1st, 1997 had a CD4 count of 650 cells/μl and an undetectable viral load of <50 copies/ml.

Using the peptide panel to stimulate cells from HTM 305 we would have selected the peptides listed in Table I. These 29 peptides include epitopes previously identified as

being restricted to 5 of the 6 MHC class I alleles expressed by this individual and included 11 epitopes within Gag gene products, 9 within Pol, 5 within Nef, and 4 within the Env gene products.

Data generated using the matrix ELISPOT identified 57 potential peptide candidates whose immunogenicity once verified in a second experiment was confirmed for 10 peptides (Table II)(Fig 1). The same previously characterized epitope was present in two overlapping peptides tested in four cases (Table II, yellow boxes)(Fig 2 A-D), stimulating with equal intensity, and likely are stimulating the same T cells. Some peptides may contain more than one epitope as is seen for pol4285 where a response to either AIFQSSMTK or SMTKILEPFR (or both) may be responsible for the number of SFC/10^6 PBMC observed (Fig2 E). The only way to confirm this is to synthesize individual optimal epitopes and test them separately. Also, despite the 10 aa overlap between peptides pol4285 and 4286, they do not contain the same A11 restricted epitope. Rather pol4286 contains a B35 restricted epitope (Fig2 E).

From the verification experiment if one adds up the number of SFC/10^6 PBMC to individual peptide and averages responses likely targeting the same epitope, HTM 305 responds to a total of 10 or 11 different epitopes in 10 peptides with a cumulative magnitude of 9230 SFC/10^6 PBMCs. Of these HIV-specific responses, 8 have been previously characterized and are represented in the peptide panel strategy. Yet 2 previously uncharacterized peptides were identified by the matrix strategy that would have been overlooked using peptide panels (Table II). One of these responses is to a peptide in the Gag gene product and the other is within Vif, a member of the accessory proteins. Given that immune response to the accessory genes are less well characterized

than for other gene products, it is likely that one would miss identifying several specificities by selecting peptide panels based on previously reported data. Thus approximately 20% of the HIV specific immune responses in HTM 305 would be overlooked by screening this subject's PBMC using the peptide panels strategy.

The peptide panel generated for HTM 310 is presented in Table III. The panel contains a total of 20 potential peptides restricted to 5 of the 6 MHC class I alleles expressed by this individual, of which 8 localize to the Gag gene products, 6 to Pol, 4 to Nef, and 2 to Env gene products. Sixty-four peptides were identified as potential candidates in a peptide pool matrix ELISPOT assay and their stimulatory capacity was verified in a confirmatory ELSIPOT assay. Of the 64 peptides tested 10 peptide responses were confirmed (Fig 3); these 10 responses had a cumulative magnitude of 9485 SFC/10^6 PBMC. In contrast to the results seen in HTM 305, there were no responses to epitopes common to two overlapping peptides among the HTM 310 responses (Table IV). Thirty percent of the responses were directed to peptides derived from the Pol gene products, and the remaining 7 responses targeted Gag gene products. Surprisingly, of the 10 identified responses, only 3 were previously described. Peptide pol 4300 contains ILKEPVHGV restricted by HLA-A68, peptide gag 5026 contains KAFSPEVIPMF restricted by HLA-B57 and pol 4296 contains QIYPGIKVR restricted by the HLA-A3 supertype of which HLA-A11 is a member (Table IV). Only 1 of these 3 would have been included if a peptide panel had been used to screen this individual (Table III). The 7 previously undescribed HIV specific immune responses in HTM 310 would also have been overlooked using the peptide panels screening method.

5.5 Discussion:

In this report we aimed to describe the feasibility and advantages of the "matrix with verification" approach over the traditional "peptide panel" ELISPOT assay. The peptide pool matrix approach frequently required larger cell numbers than did the peptide panels strategy, and cells for testing had to be frozen in two aliquots. The peptide pool matrix was more laborious than the alternate method in its initial set up and the fact that two experiments needed to be performed to identify responses. Despite these drawbacks, the matrix with verification approach was feasible and improved our ability to describe the breadth, magnitude and specificity of cells that secrete IFN-γ in response to HIV peptides. It is clear from the data presented here that information on the HIV specific immune response may be overlooked with the use of peptide panels. The approach taken here for assessing HIV-specific responses overcomes limitations inherent in selecting peptide panels as stimuli to describe the frequency and specificity of HIV-specific cells in HIV infected individuals. For example, previously undescribed epitopes can be identified without the bias introduced due to selecting stimuli based on MHC class I restriction. Responses were identified to 10 or 11 specificities for HTM 305, of which 2 were previously uncharacterized, and 10 specificities for HTM 310 of which 7 were not previously described.

Using the peptide pool matrix approach together with other methodologies it is possible to obtain further information on HIV-specific immune responses. For example pol4285 may contain more than 1 epitope recognized by HTM 305. To determine whether this is the case it would be necessary to test optimal epitopes separately to determine whether both are stimulatory. MHC class I allele restriction can be verified or

determined for novel recognition specificities using peptide pulsed EBV lines matched with responder cells for single HLA alleles. Induction of secretion of IFN-γ in responder cell, measured by flow cytometry, occurs when peptide is presented in the context of the correct HLA allele[7,10]. The minimal sequence that stimulates responder cells with the highest dilution in an ELISPOT assay identifies an optimal epitope.

Several groups have reported that individuals in primary infection respond to different epitopes than subjects observed in the chronic phase of HIV infection[6,8,12,14]. It is therefore reasonable to speculate that different populations of HIV infected patients may have different immunodominance patterns of HIV recognition. Thus subjects in primary infection, long term non progressors, slow progressors and subjects that are exposed to HIV yet uninfected may respond to different regions of the virus than subjects in chronic infection. Given the fact that peptide panels are designed largely using data reported on chronically infected subjects, it is likely that one would miss a great deal of information by using this approach to study other populations. It is therefore certain that the matrix approach will be integral in assessing immune responses that may be associated with protection in these populations.

While only 2 of the HIV specific responses were previously undescribed in HTM 305, as many as 7 of 10 were uncharacterized for HTM 310 despite the fact that they have both been infected for a similar duration. A plausible explanation for this difference may be related to the fact that HTM 310 began aggressive HAART 45 days post-infection, while HTM 305 remained largely untreated throughout follow up. We have shown previously[5,6] that early initiation of treatment can stabilize the breadth, magnitude, specificity and hierarchy of the immune response, whereas in untreated

individuals shifts in the pattern of effector responses occurs in the first year of infection with reduction in breadth and maintenance of the magnitude of the HIV-specific response. Thus the immune responses identified in HTM 310 may reflect the preserved effector activity she possessed in primary infection due to the immune protective effects of HAART. The identification of 7 previously undescribed peptide reactivities in PBMC from HTM 310 may reflect the fact that the immunodominance pattern of HIV recognition is different in primary infection than in chronic infection and that this pattern has been maintained by successful HAART since its initiation in April 1997. Comparison of immune responses in the first year of follow up with those presented here would formally address the question as to whether continuous HAART that suppresses viral replication can maintain HIV-specific immune responses for 5 or more years. Nevertheless, identification of previously undescribed peptides reinforces the advantages of the matrix approach in the analysis of populations that are not treatment naïve chronically infected individuals.

Despite 6 years of untreated infection, HTM 305 still possesses 2 uncharacterized specificities. Recently a few studies have emerged that identify a few epitopes that are targeted within the HIV accessory gene products such as Vpr[7], Vpu[1],Vif, Rev and Tat[2], yet is clear that a deficit still exists in our understanding of the role of immune responses that target these regions. Given the paucity of characterized epitopes in these accessory proteins, it is likely that the HIV peptide pool matrix approach will be integral in identifying immune responses in these uncharted regions. Of note is that one of the newly described peptides was directed against an epitope within the Gag gene product. Although responses to peptides derived from this gene product are well characterized this

does not preclude the identification of responses that have not yet been characterized perhaps due to shifting immunodominance patterns.

One of the greatest obstacles in mapping the immune response to HIV is the high mutability of the virus, and consequently potential escape of CTL epitopes from recognition. As the virus evolves to incorporate mutations, dominant responses may be lost and replaced by subdominant responses. Previously undescribed subdominant responses may be uncharacterized and overlooked with peptide panels. Using the peptide pool matrix approach can not only map the immune response but also partially monitor the evolution of the response over time in response to changes in the virus. The high mutability of the virus has another consequence that relates to describing the true breadth of the HIV-specific immune response. The use of peptide sets based on reference or consensus clade B HIV sequences rather than autologous virus likely underestimates the breadth magnitude and complete description of the specificity of immunity to HIV. However, until it is possible to work with autologous virus sequences, the peptide pool matrix strategy based on standard viral sequences provides several advantages over describing HIV specific immunity using selected peptide panels.

Figure 1

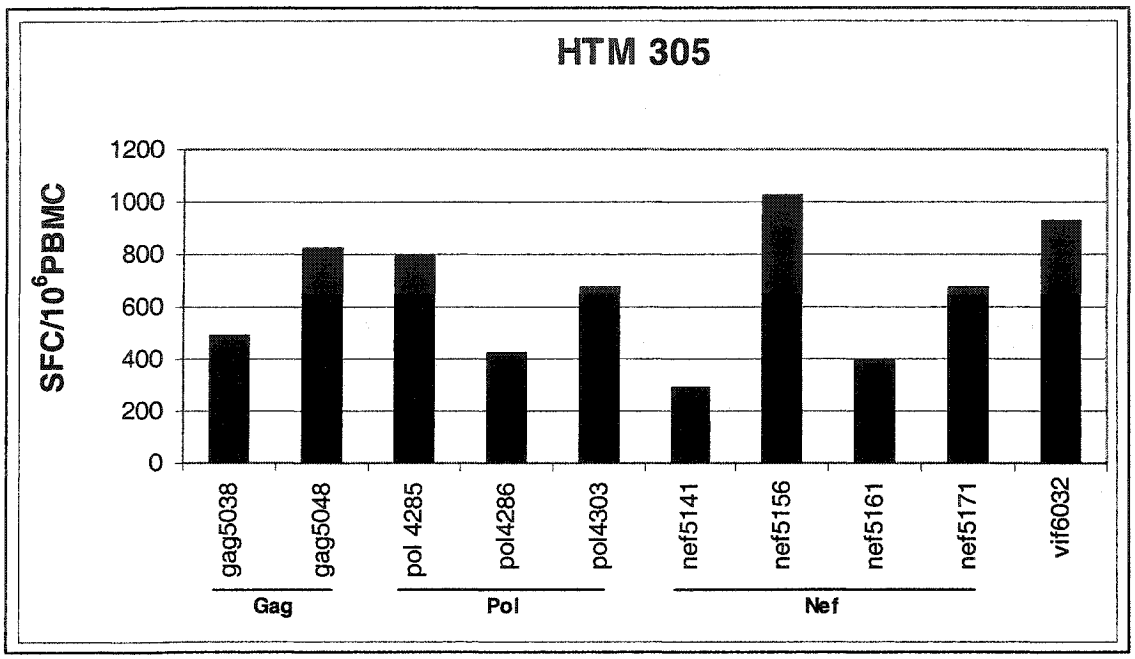
HTM 305
SFC/10^6PBMC
1200
1000
800
600
400
200
0
gag5038
gag5048
pol 4285
pol4286
pol4303
nef5141
nef5156
nef5161
nef5171
vif6032
Gag
Pol
Nef

Figure 2

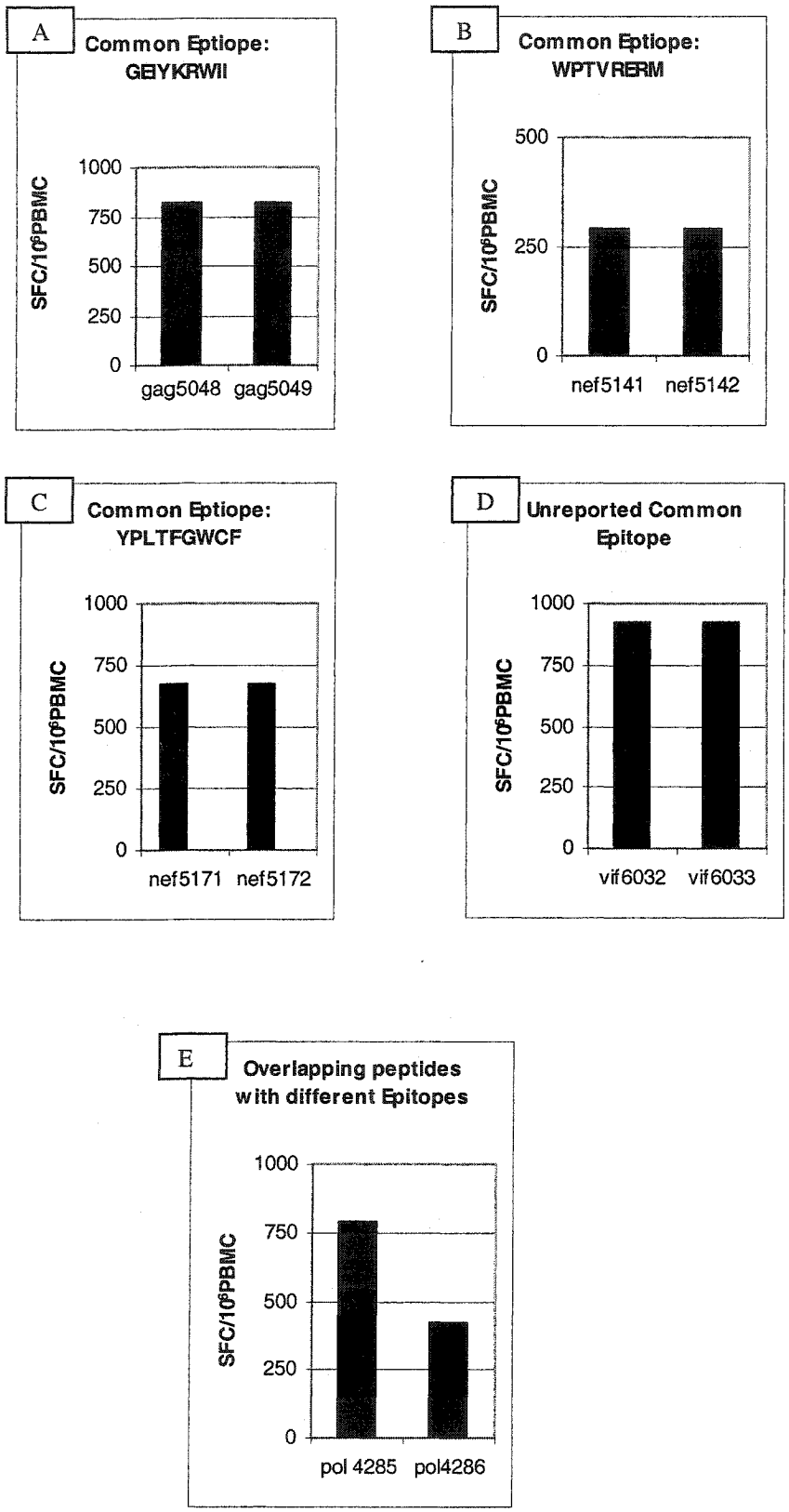
A
Common Eptiope:
GEIYKRWII
SFC/10⁶PBMC
1000
750
500
250
0
gag5048
gag5049
B
Common Eptiope:
WPTVRERM
SFC/10⁶PBMC
500
250
0
nef5141
nef5142
C
Common Eptiope:
YPLTFGWCF
SFC/10⁶PBMC
1000
750
500
250
0
nef5171
nef5172
D
Unreported Common
Epitope
SFC/10⁶PBMC
1000
750
500
250
0
vif6032
vif6033
E
Overlapping peptides
with different Epitopes
SFC/10⁶PBMC
1000
750
500
250
0
pol 4285
pol4286

Figure 3

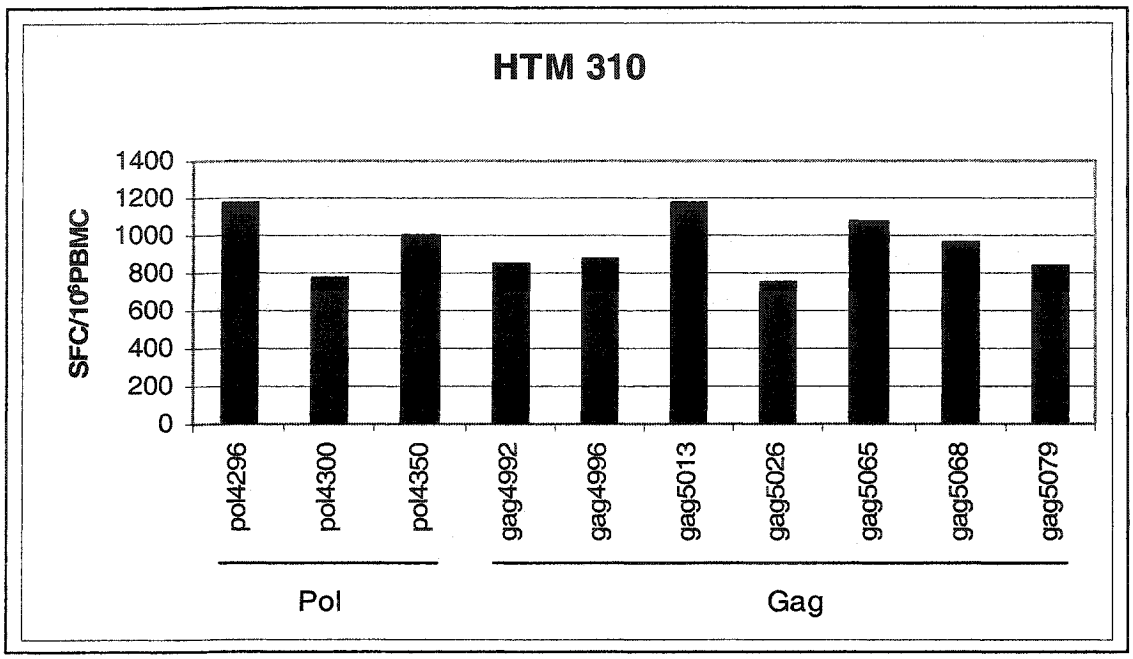
HTM 310
SFC/10^6PBMC
1400
1200
1000
800
600
400
200
0
pol4296
pol4300
pol4350
gag4992
gag4996
gag5013
gag5026
gag5065
gag5068
gag5079
Pol
Gag

Table I. Peptide Panel for HTM 305

HTM 305		
	p17(84-92)	TVA**TLYCVHQRI**DVK
	p24(349-359)	LEEMMT**ACQGVGGPGK**ARV
	RT(313-321)	WKGSP**AIFQSSMTK**I
A11	nef(73-82)	P**QVPLRPMTYK**AAVDLSHFL
	nef(84-92)	KA**AVDLSHFLK**EKGGLEGLI
	RT(507-519)	TY**QIYQEPFKNLKTG**
	gp120(36-46)	EKLW**VTVYYGVPVWK**EATTT
	gp41(584-591)	**ERYLKDQQLL**GF
A24	p24(296-306)	F**RDYVDRFYKTL**RAEQASQD
	p17(28-36)	LRPGGKK**KYKLKHIV**
	p17(24-32)	LRP**GGKKKYKLK**HIV
	p17(74-82)	GSE**ELRSLYNTV**ATL
	p24(127-135)	NPPIPVG**EIYKRWII**LGLNK
B8	p24(197-205)	VQNANP**DCKTILKAL**GPAAT
	RT(18-26)	D**GPKVKQWPL**TEEKI
	gp(586-593)	ER**YLKDQQLL**GF
	nef(13-20)	GGKWSKSSVVG**WPTVRERM**R
	nef(90-97)	KAAVDLSH**FLKEKGGL**EGLI
	p17(36-44)	LKHIV**WASRELERF**A
	p17(124-132)	AAGTG**NSSQVSQNY**
	p24(122-130)	N**PPIPVGEIY**KRWIILGLNK
	RT(107-115)	KKSV**TVLDVGDAY**GS
B35	RT(118-127)	**VPLDEDFRKY**TAFTI
	RT(156-164)	WKG**SPAIFQSSM**TKI
	RT(175-183)	KQ**NPDIVIYQY**MDDL
	RT(293-301)	**IPLTEEAEL**ELAENR
	RT(432-440)	YQLEK**EPIVGAETF**YV
	nef(68-76)	EEEEVG**FPVTPQVPL**RPMTY
Cw4	none	
Cw7	gp(586-598)	EKLWVT**VYYGVPWKEA**

Table II. Matrix Analysis for HTM 305

HLA: A11, A24, B8, B35, Cw4, Cw7

Peptide Designation	Matrix		Verif	Peptide Sequence	Characterized Epitope	HLA Restriction
gag5038	211	1475	491	DRVHPVHAGPIAPGQ	*none reported*	
gag5048	566	471	825	NPPIPVGEIYKRWII	GEIYKRWII	B8
gag5049	481	1475	825	PVGEIYKRWIILGLN	GEIYKRWII	
pol4285	204	1141	791	SPAIFQSSMTKILEPFRKQN	AIFQSSMTK	A11
					SMTKILEPFR	A3 supertype*
pol4286	359	1141	425	KILEPFRKQNPDIVIYQYMD	NPDIVIYQY	B35
pol4303	703	625	675	QWTYQIYQEPFKNLKTGKYA	IYQEPFKNL	A11
nef5141	232	458	291	SVVGWPTVRERMRRA	WPTVRERM	B8
nef5142	386	1025	291	WPTVRERMRRAEPAA	WPTVRERM	
nef5156	291	941	1025	PVRPQVPLRPMTYKA	QVPLRPMTYK	A11
nef5161	280	525	391	HFLKEKGGLEGLIYS	FLKEKGGL	B8
nef5171	195	525	675	PGPGIRYPLTFGWCF	YPLTFGWCF	B35
nef5172	158	225	675	IRYPLTFGWCFKLVP	YPLTFGWCF	
vif6032	439	442	925	IPLGDAKLVITTYWG	*none reported*	?
vif6033	296	925	925	DAKLVITTYWGLHTG	*none reported*	

putative optimal epitope

potential optimal epitope

peptide overlap, no common epitope

peptide overlap with a common epitope

**HLA-A11 is one of the alleles included in the HLA-A3 supertype*

Table III. Peptide Panel for HTM 310

HTM 310		
	p17(84-92)	TVA**TLYCVHQRI**DVK
	p24(349-359)	LEEMMT**ACQGVGGPGK**ARV
	RT(313-321)	WKGSP**AIFQSSMTK**I
A11	nef(73-82)	P**QVPLRPMTYK**AAVDLSHFL
	nef(84-92)	KA**AVDLSHFLK**EKGGLEGLI
	RT(507-519)	TY**QIYQEPFKNLKTG**
	gp120(36-46)	EKLW**VTVYYGVPVWK**EATTT
A68	RT(364-372)	N**DVKQLTEAV**QKITT
	RT(434-447)	P**IVGAETFYVDGAAN**
	p24(131-140)	**KRWIILGLNK**IVRMYSPTSI
B27	gp(786-795)	IVELL**GRRGWEVLKY**WWNLL
	nef(105-114)	HSQ**RRQDILDEWI**YHTQGYF
	p24(15-23)	VHQA**ISPRTLNAW**VKVVEEK
	p24(30-37)	NAWVKVVEE**KAFSPEVIPMF**
	p24(176-184)	TLRAE**QASQDVKNW**MTETLL
B57	Rt(374-383)	VQ**KITTESIVIW**GKTP
	RT(244-252)	P**IVLPEKDSW**TVNDI
	nef(116-125)	LWIYHTQG**YFPDWQNYT**PGP
	p24(108-117)	GSDIAGT**TSTLQEQIGW**MTN
Cw1	p24(36-43)	AFSPE**VIPMFSAL**SEGATPQ
Cw6	none	

Table IV. Matrix Analysis for HTM 310

HLA: A11, A68, B27, B57, Cw6, Cw7

Peptide Designation	Matrix		Verif	Peptide Sequence	Characterized Epitope	HLA Restriction
pol4296	302.5	312.5	1170	WASQIYPGIKVRQLCKLLRG	QIYPGIKVR	A3 supertype
pol4300	312.5	312.5	780	NREILKEPVHGVYYDPSKDL	ILKEPVHGV	A68
pol4350	312.5	272.5	1000	EGAVVIQDNSDIKVVPRRKA	*none reported*	
gag4992	312.5	722.5	850	YKLKHIVWASRELER	*none reported*	
gag4996	182.5	722.5	870		*none reported*	
gag5013	272.5	722.5	1180	KKAQQAAADTGHSNQ	*none reported*	
gag5026	312.5	162.5	755	EKAFSPEVIPMFSAL	*none reported* KAFSPEVIPMF	 B57
gag5065	302.5	722.5	1080	LLVQNANPDCKTILK	*none reported* *none reported*	
gag5068	272.5	722.5	960	ILKALGPAATLEEMM	*none reported*	
gag5079	272.5	182.5	840	MMQRGNFRNQRKIVK	*none reported*	

putative optimal epitope

potential optimal epitope

Figure 1. Total HIV specific effector responses generated by HTM 305. Bar graphs represent the peptide specific responses, from the confirmatory experiment, in SFCs per million PBMCs (SFCs/10^6 PBMCs). Following the matrix ELISPOT, using pools of overlapping peptides spanning all HIV-expressed gene products, peripheral blood mononuclear cells (PBMCs) from subject HTM 305 were stimulated with the peptide panel, 57 peptides, identified as candidate stimuli from the matrix ELISPOT. The *x*-axis indicates, for each bar, the 10 peptides that induced responses in the confirmatory experiment.

Figure 2. Comparison of the magnitude of the HIV specific response for overlapping peptides for HTM 305. Bar graphs represent the magnitude in SFCs/10^6 PBMCs for each of the two characterized overlapping peptides that were tested in the confirmatory experiment. Overlapping peptides containing the same previously characterized epitope (A-C), containing an undescribed possibly common epitope (D), and two potentially distinct epitopes (E) are presented here.

Figure 3. Global HIV specific effector activity for HTM 310. Bar graphs depict the peptide specific responses, from the confirmation experiment, in SFCs/10^6 PBMCs. The *x*-axis represents, for each bar, the 10 peptide specific responses that were identified in the confirmatory experiment from the 64 potential responses that emerged from the matrix ELISPOT.

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Chapter 6: Comprehensive Analysis of HIV-Specific Effector Responses in Chronic HIV Infection

The chronic phase of infection follows PI as patients enter their first year of infection. While initiation of therapy in early PI appears to preserve HIV specific immune responses, patients who do not begin effective treatment early enough (chapter 3) or who elect to remain untreated (chapter 4) appear to respond to the virus differently than patients in early PI (chapter 2). Given that the hierarchy of the immune response changes considerably during the first year of infection (chapter 4), it is clear that viral replication and changes in the viral sequence induce significant alterations in the manner in which the host responds to the virus. Thus to grasp the impact of viral replication on the host response it is essential to characterize the strength, breadth, and specificity of the immune response at different stages of disease.

Reports intended to characterize the immune response in chronic infection have been greatly limited by the use of peptide panels, given the data presented in chapter 5, it is clear that a great deal of information may be overlooked with the use of this strategy. Thus new strategies such as the matrix (chapter 5) now allow for the dissection of the true pattern of the immunodominance of the antiviral response in an unbiased fashion.

CHARACTERIZATION OF THE BREADTH, MAGNITUDE, AND SPECIFICITY OF THE HIV-SPECIFIC EFFECTOR RESPONSE IN 24 HLA-A2 UNTREATED CHRONICALLY INFECTED SUBJECTS

Running Title: HIV-specific effector activity in chronic infection

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6.1 Abstract

The asymptomatic phase of the disease caused by the human immunodeficiency virus is characterized by a relatively stable plasma viremia set point, which is maintained through a balance between the rate of viral production, and the rate of viral clearance by cells of the immune system. In this report, we have employed peptide pool-matrices to screen peripheral blood mononuclear cells (PBMC) from chronically HIV infected HLA-A2 positive subjects for HIV-specific immune responses using an ELISPOT assay. The matrix ELISPOT overcomes limitations inherent in selecting peptide panels as stimuli to describe the frequency and specificity of HIV-specific cells in HIV infected individuals. Responses were identified to 111 specificities, 41 (37%) of which were previously not characterized in the literature. It appears that chronically infected subjects display a heterogeneous HIV-specific response with respect to breadth and magnitude. HIV specific effector responses directed to HIV gag gene products (p17, p24, and p15) predominated with responses to nef being the second most frequently recognized gene product. In twelve individuals in which antigen specific IFN-γ secretion and CTL activity to the same HIV peptide were evaluated these two responses were frequently discordant.

6.2 Introduction:

Infection with the human immunodeficiency virus (HIV) can be divided into three phases: primary infection often associated with an acute seroconversion syndrome and a spike in viral load that falls as HIV-specific cytotoxic T lymphocytes (CTL) are induced, an extended asymptomatic period that lasts from 2-10 years and a late phase usually lasting less than 2 years in untreated patients beginning with diagnosis of acquired immunodeficiency syndrome (AIDS)[29]. CTL play an integral role in the control of viremia in HIV infection. In acute infection, CTL induction is temporally associated with a reduction in plasma viremia[8,22]. In late stage disease, as CTL function declines, a rise in viremia occurs[21,29]. Moreover, selection for mutations within sequences recognized by CTL provides evidence that these immune responses exert pressure that suppresses viral replication[17,20,24,26,30].

The asymptomatic phase of the disease is characterized by a relatively stable plasma viral load set point, which is maintained through a balance between the rate of viral production, and the rate of viral clearance by cells of the immune system[29]. Studies in an animal model for HIV infection, i.e. macaques infected with simian immunodeficiency virus (SIV) demonstrate that CD8+ T cell depletion in macaques, during this phase of the disease, results in uncontrolled viremia and rapid disease progression[19,33]. As many as 1-2% of circulating cells are HIV-specific as determined by staining with HIV peptide tetramer reagents[25].

Broad and intense CTL responses have been observed in the chronic phase of HIV infection[14]. Several studies have characterized the breadth, magnitude and specificity of HIV-specific immune responses using panels of peptides selected based on

their being restricted to one of the MHC class I alleles expressed by the person being tested[2-4,14,15,28]. Availability of HIV peptide sets corresponding to all expressed HIV genes and high throughput assays such as the enzyme-linked immunospot assay (ELISPOT) assay have permitted the design of screening strategies for HIV-specific immune responses that are unbiased with respect to previously described MHC class I restricted peptides[1,7,10]. Using such approaches should provide a more complete picture of the number and location of peptides recognized as well as of the frequency of cells recognizing these peptides in HIV infected persons than was previously possible. This type of analysis is a first step towards identifying the regions of the virus that may be important for control of viral replication in the chronic phase of HIV disease and may be useful for identifying the correlates of protection against progression. In this report, we have employed peptide pool-matrices to screen peripheral blood mononuclear cells (PBMC) from chronically HIV infected HLA-A2 positive subjects for HIV-specific immune responses using an ELISPOT assay. This approach assessed the breadth, magnitude and specificity of PBMC secreting IFN-γ in response to peptide stimuli derived from all expressed HIV genes.

6.3 Methods:

Study population: Twenty-four (24) HIV infected HLA-A2 positive subjects in the chronic phase of infection who were naïve to highly active antiretroviral therapy (HAART) were analyzed. The institutional review board of the study site approved this study and all participants signed informed consent. Viral load measurements were performed using the HIV RNA b-DNA version 2.0 assay (Chiron, Corp. Emeryville, CA) with a lower limit of detection of 500 HIV RNA copies/ml of plasma.

Cells: PBMCs were isolated from blood by density gradient centrifugation (Ficoll-Paque, Pharmacia Upsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma, St Louis MO) with 90% fetal calf serum (FCS., Canadian Life Technology, Burlington, On).

Flow Cytometry: CD3 FITC, CD4 APC and CD8 PE reagents (Becton Dickinson, Mississauga, Ontario) were employed to assess absolute CD4 and CD8 T cell numbers.

HLA typing: Subjects were typed for major histocompatibility complex (MHC) class I antigen expression by the amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI)[9]. Genomic DNA for molecular HLA-typing was prepared from Epstein-Barr virus (EBV) transformed B cell lines using the QIAamp DNA blood kit (Qiagen Inc., Mississauga, ON).

Design of Peptide Matrices: The HIV peptide sets used for stimulation were 15 amino acids (aa) with 11 aa overlaps (Gag, Env, Nef, Tat, Rev, Vpr, Vpu, Vif) or 20 aa with 10 aa overlaps (Pol). The peptides were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Lyophilized peptides (n=621) spanning all HIV-1 gene products were dissolved to a final concentration 10 mg/ml in DMSO (Sigma) and stored at -70°C. These included 100 Pol 20-mers and 123 Gag 15-mers corresponding to the HIV-1_{HXB2R} clade B isolate, 49 Nef, 27 Rev, 23 Tat, 46 Vif, 22 Vpr and 19 Vpu 15-mers corresponding to consensus HIV (Vpu) or consensus clade B sequence (Nef, Rev, Tat, Vif, Vpr) and 212 Env-15-mers corresponding the HIV_{MN} clade B isolate. Pools containing 2-15 peptides were prepared and organized into matrices of Gag, Pol, Nef, Env and accessory gene peptide pools such that each peptide was present in two pools within each peptide matrix.

ELISPOT assay for single cell Interferon-γ (IFN-γ) release: IFN-γ secretion by HIV-specific cells was quantified using the ELISPOT assay. Cells were plated at 6 $x10^4$ to 10^5 PBMCs per well and were stimulated in round-bottomed plates with the peptide pools for 3 hours. The final concentration of each peptide within a pool was 2 to 4 μg/ml. Media alone was used as a negative control and anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) was used as a positive control stimulus. Results are expressed as spot forming cells per million PBMC (SFC/10^6 PBMC) following subtraction of negative controls. Negative control stimulation produced less than 5 spots per well in greater than 90% of experiments. The average number of spots in the negative control wells was 3.13 ± 3.12. In experimental wells the signal was considered positive if at least 10 spots per

well were present and the number of spots was at least 3-fold greater than the negative control wells. The identity of IFN-γ secreting cells as CD8+ was confirmed by the reduction of SFC numbers following depletion of CD8+ cells with magnetic beads (Dynal, Lake Success, NY).

Confirmation of Peptide Specificity: The stimulatory capacities of candidate stimulatory peptides identified in the peptide-pool matrix ELISPOT assay were confirmed in a second experiment with cells from the same time point stimulated with individual peptides that were common to two stimulatory peptide pools in the initial ELISPOT screen. Confirmation peptide panels consisted of 4-54 potential peptide candidates. For single peptide verification experiments, cells were plated in triplicates with 4 μg/ml of individual candidate peptides. The same criteria were used in the verification assay as in the original peptide pool matrix experiment to identify positive responses. For a subset of experiments an immunodominant HLA-A2-restricted EBV-derived peptide (NLVPMVATV) was also used as positive control stimuli.

Cytotoxicity Assay: CTL lines were established using 10^6 PBMC stimulated with 10μM HLA-A2 restricted optimal peptides Gag 77-85 (SLYNTVATL) and RT 476-484 (ILKEPVHGV) in the presence of 40ng/ml of interleukin-7 (IL-7). Cells were grown in RPMI 1640 (GIBCO, Grans Island, NY) containing 10% FCS (Montreal Biotech Inc., Montreal, QC, Canada), 2 mM L-glutamine (ICN Biomedical Ltd, Aurora, OH), 50 IU/ml penicillin (ICN), 50 mcg/ml streptomycin (ICN) and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO). Following a 3-day stimulation in the presence of the peptide

alone, cells were maintained at a final concentration of 200 IU/ml of interleukin-2 (IL-2) for an additional 18 days. On day 21 these cells were used as effectors in a 4-hour chromium release assay. Targets were autologous Epstein Barr virus -transformed B cell lines labeled with 200 μCi $Na_2{}^{51}CrO_4$ overnight and washed three times before plating. Targets were plated at 5 x 10^3 cells/well and pulsed with peptide at a concentration of 50 μg/ml for 1 hr before adding effector cells. Effectors were added at an effector to target ratio (E:T) of 20:1, 10:1 and 5:1. Maximal release was determined from wells containing target cells treated with 1% NP-40 and spontaneous release from wells containing target cells incubated with media alone. Percent specific lysis was calculated using the equation: ((experimental release - spontaneous release)/(maximal release – spontaneous release)) x100. Percent specific lysis was considered positive if > 10% over background with at least 2 E:T .

Statistical Analysis: The Student T-test was utilized in order to assess the significance of differences in the magnitude of responses to gene products and the distribution of viral load, CD4 and CD8 T cell numbers among subjects who did or did not possess lytic activity. Pearson linear correlation analyses were employed to establish the strength of the relationship between HIV specific effector parameters (breadth, magnitude, and CTL activity) and several clinical parameters (viral load, CD4 and CD8 T cell numbers). P-values of less then 0.05 were considered significant.

6.4 Results:

Study population: A description of the study population is provided in Table 1. One of the 24 subjects was female, the median age of the sample was 34 (range 14-44) yrs. Twenty-one subjects were infected through sexual exposure, 1 via intravenous drug use, and 2 were hemophilia patients infected through the transfusion of contaminated blood products. The median duration of infection was 3.3 (range 1-11) yrs. Patients manifested median CD4 counts of 612 (range 525-1140) cells/mL, median CD8 T cell numbers were 1139 (range 689-2046) cells/mL, and the median viral load was 943 (range 499-22600) HIV RNA copies/ml of plasma. For all viral loads below the limit of detection, a value of 499 copies/mL was assigned. All subjects expressed HLA-A2; no other HLA allele appeared over-represented in the study population.

HIV Specific Effector Responses: HIV peptide pool matrices corresponding to peptides spanning all expressed HIV genes were employed to assess the pattern and strength of the HIV specific response in PBMC from the study population. Subjects responded to a median of 5 (range 0 to 13) peptides, (Fig1 A) with a median magnitude of 1412 (range 0-5833) SFC/10^6 (Fig1 B). Each subject recognized an average of 2 gene products. A significant correlation was observed between the breadth and the magnitude of the response (r^2=0.7; p=0.00, Pearson linear correlation) (Fig1 C).

Immune responses for individual gene products were normalized so that both the magnitude and the breadth were assessed proportionally to the size of the individual gene products within the viral sequence. According to the Los Alamos database p17, p24, p15, protease, reverse transcriptase (RT), integrase, Vif, Vpr, Tat, Rev, Vpu, gp120, gp41and

Nef represent 4.5%, 7.6%, 4.2%, 5.3%, 18.1%, 9.6%, 6.3%, 3.2%, 3.35%, 3.35%, 2.7%, 17.1%, 11.1% and 6.65% of the protein sequence, respectively. Note that Gag p55 includes Gag p17, p24 and p15; Pol includes protease, RT and integrase; and Env gp160 includes gp 120 and gp41. Despite the fact that Gag p55 only represents 16.3% of the entire HIV amino acid sequence, peptides derived from this gene product were targeted more frequently (35 of 88 [46%] of all peptides recognized by this group of chronically infected subjects were derived from Gag p55, Fig1 A) and with a higher magnitude (46419 of 80075 SFC/10^6 PBMC [57%] of all HIV-specific cells recognized Gag p55, Fig 1B) than any other viral gene product ($p<0.001$ for both breadth, χ^2test, and magnitude, unpaired T-test) (Fig1 D and E). RT represents 18% of the entire HIV amino acid sequence. Eight percent of the cumulative magnitude and 18% of the breadth were directed against peptides derived from this protein (Fig1 D and E). Sixteen percent of the breadth and 14.4% of the magnitude of the response to all expressed HIV genes were directed at peptides derived from the Nef protein, which only spans 6.65% of the protein sequence (Fig1 D and E).

Interestingly, PBMC from 8 of 24 (33%) individuals in this chronically infected population recognized accessory gene products. To date, accessory gene products have been understudied with respect to the mapping of HIV derived CTL epitopes. The approach taken here permitted the identification of several responses directed to accessory gene products including previously undescribed epitopes (Fig1 D and E). Surprisingly, 7.8% of the breadth and 6.1% of the magnitude were directed at sequences derived from the Rev protein, which represents as little as 3.35% of the total HIV sequence (Fig1 D and E). Similarly, 4.7% of the breadth and as much as 10.8% of the

cumulative magnitude of the HIV-specific immune responses in these 24 subjects recognized peptides derived from the Vpr sequence, representing 3.2% of the HIV aa sequence (Fig1 D and E). The remaining gene products contributed minimally to the breadth and magnitude of the immune response.

Given the heterogeneity of the immune response to HIV, we sought to establish whether a correlation existed between any clinical parameters and the strength (Figure 2A, C, E) or breadth (Fig 2B, D, F) of the immune response. No correlation was observed between the absolute CD4 T cell number (Fig2 A, B), absolute CD8 T cell count (Fig2 C and D), or viral load (Fig2 E and F) and either the breadth or the magnitude of the immune response to HIV (p=not significant for all comparisons).

Of the subjects tested by ELISPOT assay, 12 of 24 (50%) recognized the Gag 77-85 epitope an average magnitude of 330±368 SFC/10^6 PBMCs and 5 of 24 (20%) recognized RT 476-484 with an average magnitude of 284±191 SFC/10^6 PBMCs. Others have shown that despite significant proportions of HIV specific tetramer positive cells present during the chronic phase of the infection, these cells may be functionally impaired in their ability to proliferate, express perforin and, lyse targets[18]. Given that several subjects had detectable levels of cells that responded to these HLA-A2 restricted epitopes by secreting IFN-γ, we investigated whether these cells could also develop lytic activity specific for these epitopes.

For these experiments a subset of 12 individuals were tested for both secretion of IFN-γ by ELISPOT assay and indirect CTL activity to Gag 77-85 and RT 427-484. As shown in Table 2, 8 of 12 (75%) subjects tested recognized Gag 77-85 by secreting IFN-γ while only 3 of these individuals mounted a CTL response directed at this epitope (Table

2). Six of 12 (50%) subjects secreted IFN-γ in responses to RT 476-484; of these 6 individuals 3 also mounted a CTL response to this peptide (Table 2). All those who mounted a peptide specific CTL response also secreted IFN-γ upon recognizing antigen. However only a subset of those having a positive peptide specific response by ELISPOT also recognized that peptide in an indirect CTL assay (Table 2). No between group differences existed for viral load, CD4 or CD8 T cell numbers between subjects that exhibited CTL specific lysis and those that did not ($p=0.8$, unpaired T-test). Additionally, no correlation was observed between CTL activity and the magnitude, or the breadth of IFN-γ secretion (data not shown). This data shows that despite the presence of antigen specific cells able to secrete IFN-γ, functional defects in antigen specific cells were nevertheless evident in this chronically infected population.

6.5 Discussion:

We mapped the breadth, magnitude and specificity of cells that secrete IFN-γ in response to HIV peptide stimulation in 24 untreated HLA-A2 subjects in the chronic phase of infection. In this report we found that chronically infected subjects display a heterogeneous HIV-specific response with respect to breadth and magnitude. HIV specific effector responses directed to HIV Gag gene products (p17, p24, and p15) predominated with responses to Nef being the second most frequently recognized gene product (Fig 1d and e). Neither the breadth nor the magnitude of HIV-specific responses in each individual correlated with viral load levels, absolute CD4 or CD8 T cell numbers. In twelve individuals in whom antigen specific IFN-γ secretion and CTL activity to the same HIV peptide were evaluated only a subset of individuals with peptide specific ELISPOT responses also had lytic activity to the same peptide.

The approach taken here for assessing HIV-specific responses overcomes limitations inherent in selecting peptide panels as stimuli to describe the frequency and specificity of HIV-specific cells in HIV infected individuals. For example, previously undescribed epitopes can be identified without the bias introduced due to selecting stimuli based on MHC class I restriction. Responses were identified to 111 specificities, 41 (37%) of which were previously not characterized in the literature. Thus 37% of specificities observed in this study are potentially new targets that may be important for the control of viral replication.

Previous reports have described broad and intense HIV specific effector activity in chronically infected patients[14]. Yet it appears that not all HIV infected subjects in the chronic phase of infection respond to HIV by developing a high frequency of PBMC

secreting IFN-γ to a broad range of peptides. Rather the pattern appears quite variable, and does not correlate with clinical parameters such as viral load, CD4 or CD8 T cell numbers. Dalod et al. described 34 chronically HIV infected subjects who responded to a mean of 5 peptides with a total intensity of 4084 SFC/10^6 cells. This is consistent with results reported here where subjects within our study also responded to a median of 5 peptides, (range 0-13 peptides); the median intensity of the HIV-specific responses was 1412 SFC/10^6 (range 0-5833 SFC/10^6SFC), lower than that reported by Dalod et al.[14]. One explanation for the lower median magnitude of HIV-specific responses seen in the population studies here may be that viral load drives the immune response as has been reported by others[12,14,27,34]. The mean viral load in the population studied by Dalod et al was 10-fold higher than that seen in our sample[14].

A number of reports suggest that polyclonal responses may be associated with better clinical outcome than narrow responses[6,11,17,30]. Subjects with monoclonal responses appear to lose the ability to control viral replication in the event that the virus escapes immune recognition. Data from our study suggests that there is no clear association between the polyclonality of the response and a number of clinical parameters that are associated with clinical outcome. Thus the individuals with only a few responses may be as effective in controlling their viral loads as subjects exhibiting up to a dozen different specificities. This suggests that viral control may be mediated by not only targeting a broad range of epitopes but also by targeting the correct specificities.

The population studied here is not representative of typical HIV disease progressors as many individuals included in this report have lower viral loads and higher CD4 T cell numbers than typical HIV disease progressors. Therefore caution should be

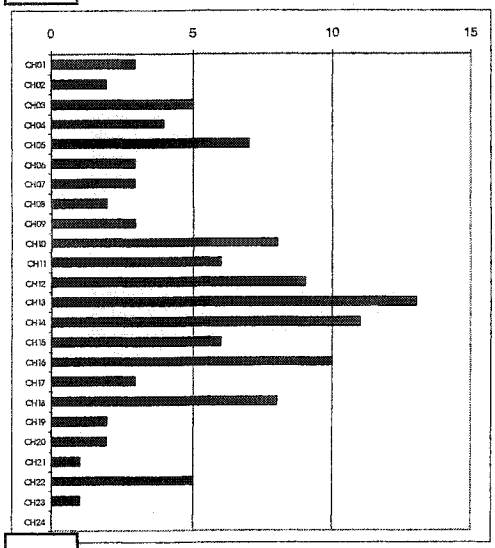
exercised in extrapolating results generated in this population to all chronically HIV infected progressors. Nevertheless it is interesting to note that in this relatively healthy population of chronically HIV infected individuals discordance between IFN-γ secretion and CTL activity to individual peptides is evident. Lytic activity was independent of viral load, CD4 and CD8 T cell counts. Thus it is unlikely that high viral loads, perhaps resulting in greater CD4 depletion, would be the cause of this impaired effector activity. Moreover, the inability to mediate this essential effector function, while HIV specific IFN-γ secretion appears functional, has been previously described[5]. The indirect CTL assays used to assess the presence of HIV peptide specific CTL activity were performed on T cell lines prepared from PBMCs stimulated with specific HIV peptides followed by a 21 day culture period. In order for these T cell lines to have antigen specific lytic activity peptide specific cells must proliferate. Absence of lytic activity may therefore be due to the predominance of terminally differentiated antigen specific cells in the individuals being tested[5,13,25]. This may be the reason that an ex vivo assay such as the ELISPOT assay and the CTL assay frequently give discordant results in the population tested. Champagne et al showed that HIV infected individuals had a skewed distribution of memory CD8+ T cell subsets characterized by the accumulation of HIV specific cells at a pre-terminally differentiated stage that was not able to efficiently develop into effector cells able to lyse targets nor to proliferate efficiently[5,13]. One can speculate that these defects may be established early in disease, during the primary phase of the infection, where HIV specific CD4 T cells are preferentially infected and depleted or anergized[16]. Several reports have described the critical nature of the preservation of HIV-specific CD4 T cell responses in order to maintain effective CD8 T cells in the context of

primary infection and long term non progressive infection[23,31,32]. Depletion of these cells during acute HIV infection can lead to an ineffective response to the virus. Thus in this study, we show that in spite of relatively high CD4 numbers, virus specific cells may already be eliminated or non functional, leading to reduced maturation, proliferation, and impaired effector activity. Thus a relatively intact immune system with high CD4 numbers may not be indicative of an effective immune response.

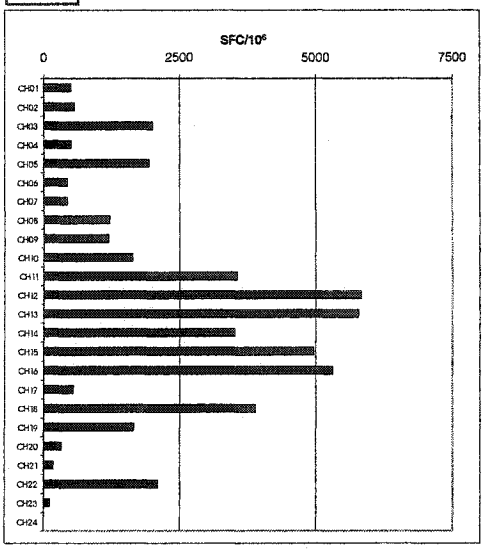
In summary, here for the first time, we present a complete picture of the immune response in 24 treatment naïve chronically infected HLA-A2 HIV+ patients. The HIV specific immune response in untreated chronic infection appears to be heterogeneous. Data presented here demonstrates that the magnitude and breadth of HIV-specific immunity are directed predominantly towards proteins derived from the gag p55 gene product. No clear relationship was discernable between clinical markers such as CD4, CD8, or viral load with respect to effector responses. Using the peptide pool matrix ELISPOT approach to assess responses directed at all expressed HIV genes identified a significant number of previously undescribed peptides, particularly in gene products poorly characterized with respect to epitope mapping such as rev and vpr. Thus it is clear that the use of this technique will be critical in allowing for the complete characterization of immune responses in populations at different stages of infection. Consequently it may now be possible to identify novel responses associated with the maintenance of viral load set point and potentially protection from progression.

Figure 1.

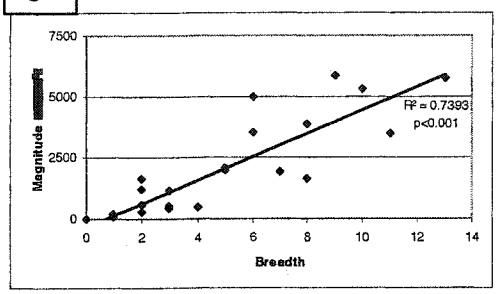
A



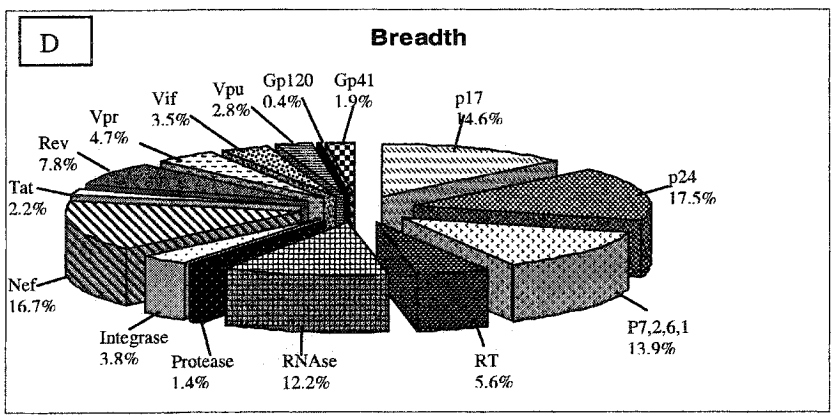
B



C



D



E

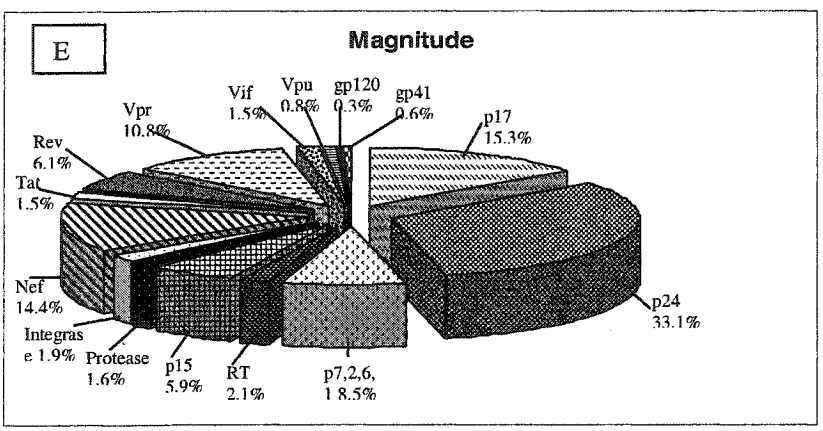


Figure 2.

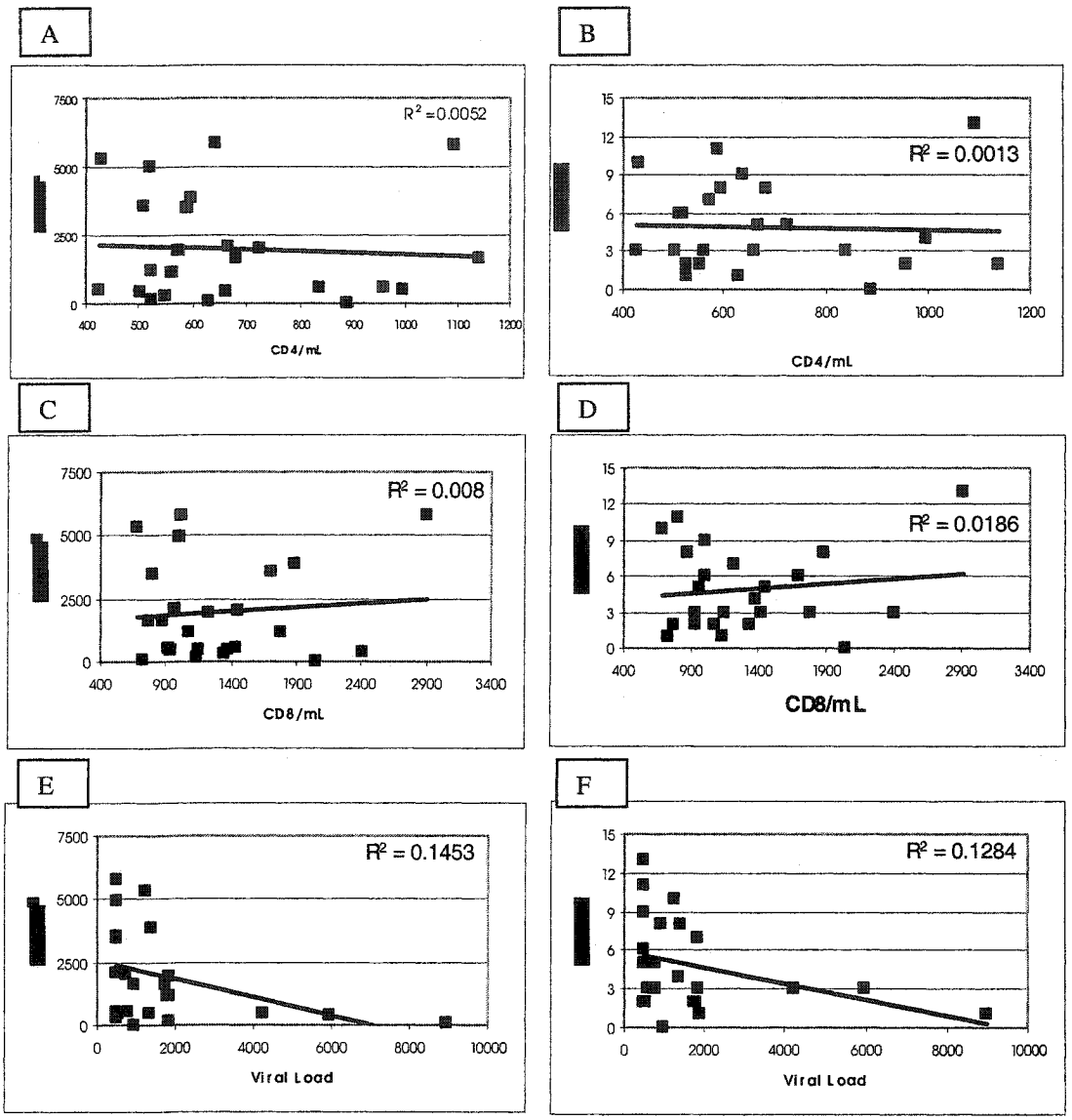

A
7500
5000
2500
0
400 500 600 700 800 900 1000 1100 1200
CD4/mL
R² =0.0052
B
15
12
9
6
3
0
400 600 800 1000 1200
CD4/mL
R² = 0.0013
C
7500
5000
2500
0
400 900 1400 1900 2400 2900 3400
CD8/mL
R² = 0.008
D
15
12
9
6
3
0
400 900 1400 1900 2400 2900 3400
CD8/mL
R² = 0.0186
E
7500
5000
2500
0
0 2000 4000 6000 8000 10000
Viral Load
R² = 0.1453
F
15
12
9
6
3
0
0 2000 4000 6000 8000 10000
Viral Load
R² = 0.1284

Table I. Descriptive Statistics

	Sex	Mode of Transmission	Age	CD4	CD8	Viral Load	A Locus		B Locus		C Locus	
MQCH01	M	MSM	34	525	935	4245	A2	A11	B14	B39	Cw7	Cw8
MQCH02	M	MSM	32	960	928	516	A2	A11	B44		Cw4	Cw5
MQCH03	M	MSM	25	725	1450	758	A2	A3	B7	B14	Cw7	Cw8
MQCH04	M	MSM	34	999	1377	1358	A2	A11	B7	B14	Cw8	Cw7
MQCH05	M	MSM	28	572	1222	1846	A2	A29	B15	B27	Cw1	Cw2
MQCH06	M	MSM	33	660	1144	564	A2	A24	B18	B44	Cw4	Cw5
MQCH07	M	MSM	26	504	2412	5952	A2	A26	B15	B38	Cw3	Cw12
MQCH08	M	MSM	44	525	1071	1792	A2		B41	B51	Cw14	Cw17
MQCH09	M	MSM	40	561	1782	1857	A2	A1	B27	B44	Cw2	Cw5
MQCH10	M	MSM	42	682	880	926	A2	A26	B35	B40	Cw2	Cw4
MQCH11	M	MSM	37	510	1700	499	A2	A26	B53	B72	Cw2	Cw4
MQCH12	M	MSM	44	638	1012	499	A2		B40	B44	Cw2	Cw5
MQCH13	M	MSM	34	1092	2912	499	A2		B27	B40	Cw2	Cw16
MQCH14	F	Transfusion	35	589	798	499	A2	A11	B27	B52	Cw2	Cw12
MQCH15	M	MSM	36	520	1000	499	A2	A26	B27	B62	Cw1	Cw3
MQCH16	M	MSM	36	529	689	22600	A2	A24	B7	B27	Cw1	Cw7
MQCH17	M	MSM	42	840	1428	776	A2	A1	B44	B57	Cw6	Cw16
MQCH18	M	MSM	29	595	1890	1414	A2	A11	B7	B35	Cw4	Cw5
MQCH19	M	Hemophiliac	14	1140	782	1736	A2		B40	B47	Cw2	Cw15
MQCH20	M	IVDU	29	552	1344	499	A2	A1	B8	B18	Cw8	Cw18
MQCH21	M	MSM	31	525	1134	1874	A2	A24	B15	B40	Cw3	
MQCH22	M	MSM	31	667	966	499	A2	A3	B14	B44	Cw8	Cw5
MQCH23	M	MSM	27	630	735	8961	A2	A33	B14	B40	Cw2	Cw8
MQCH24	M	MSM	33	891	2046	959	A2	A31	B40	B44	Cw3	Cw5

Table II. Summary of HIV specific effector activity

	Gag 77-85	Gag 77-85	RT 476-484	RT 476-484	Total Breadth	Total Magnitude
	% specific lysis	SFC/106 PBMC	% specific lysis	SFC/106 PBMC		SFC/106 PBMC
MQCH02	1	340	0	0	2	557
MQCH04	24	550	18	215	4	493
MQCH05	3	233	1	0	7	1930
MQCH06	37	228	36	150	3	433
MQCH08	6	0	2	0	2	1198
MQCH09	1	138	7	210	3	1175
MQCH11	19	115	4	0	6	3547
MQCH14	0	105	1	133	11	3498
MQCH15	2	0	11	110	6	4973
MQCH18	0	0	0	0	8	3886
MQCH20	0	113	0	208	2	313
MQCH24	2	0	4	0	0	0

Figure 1. Comprehensive analysis of the global HIV-specific effector activity. 24 chronically infected HAART naïve HLA-A2 subjects were screened for HIV specific effector responses by interferon- γ (IFN-γ) matrix ELISPOT. Bar graphs represent the breadth (A) and the magnitude in SFC per million PBMCs (SFCs/10^6 PBMCs) (B) of HIV specific activity for all subjects tested. Scatter plots (C) show the correlation between the magnitude and the breadth of the HIV-specific effector response for al 24 subjects tested. Pie chart analysis depicts the distribution of HIV specific effector breadth (D) and magnitude (E) over all HIV gene products.

Figure 2. Correlation between HIV specific effector activity and viral load, CD4 or CD8 T cell numbers. Scatter plots represent the association of the magnitude in SFCs/10^6 PBMCs (A, C, E) or the breadth (B, D, F) and CD4 T cell numbers (A, B), CD8 T cell numbers (C,D) or viral load (E, F) for all 24 subjects tested.

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Chapter 7: Summary of original findings

Several novel findings arose from the research described in this thesis.

1) Chapter 2 investigated the pattern of the HIV specific immune response in a cross sectional study of a group of 25 treatment naïve subjects in primary infection.
    a. Subjects in the first year of infection recognize more peptides than subjects in the chronic phase of infection.
    b. The immune response broadens and intensifies during the first two month after infection.
    c. PBMCs from subjects screened at a time when they were 2 months or less from infection exhibited significantly narrower responses to viral epitopes than subjects in PI infected for more than 2 months.
    d. Earlier targeting of peptides derived from the Nef gene product suggests that immune responses directed towards this viral protein may be induced earlier than responses directed to other viral gene products.

    In summary, we were able to demonstrate for the first time that there is a clear evolution in the immune response to HIV with time during PI.

2) In Chapter 3 we explored the impact on the anti-viral immune response to HIV of initiating HAART at various times from infection.
    a. Longitudinal follow-up of subjects treated at different times from infection revealed that treatment of all seronegative, and 5/11 sero-positive/detuned-negative (s+/d-) subjects maintained both the breadth and magnitude of their HIV specific effector responses despite the suppression of viral

replication. In contrast, all seropositive/detuned-positive (s+/d+), chronically infected subjects, as well as the remaining 6/11 (s+/d-) individuals experienced a significant contraction in the breadth and intensity of the HIV-specific immune response over time.

b. Preservation of the immune response was not correlated with reduced plasma viremia at the time of therapy initiation.

c. An association was observed between the preservation of HIV-specific CD4 IFN-γ secretion and maintenance of HIV specific effector activity. Gag p55 specific IFN-γ secretion by both the CD4 and CD8 T cell compartments was detected in subjects tested that were s-/d- and subjects maintaining effector responses that were s+/d- over the follow-up period. In contrast, all individuals tested who showed a narrowing and decrease in the intensity of HIV-specific immunity after treatment also exhibited a declining frequency of IFN-γ+ HIV Gag p55-specific CD4+ and CD8+ T cells. Thus it is likely that the maintenance of HIV-specific CD4+ T cell help may be an important factor in the preservation of HIV specific effector responses over time.

In this chapter, for the first time, we demonstrated that initiation of HAART beyond the acute phase of HIV infection can still have beneficial effects on the preservation of HIV specific immune responses. The deciding factor as to whether this will occur appears to be whether initiation of HAART can still rescue HIV-specific CD4+ T cell function. This work provides a rationale for using the detuned assay as a clinical tool for decision making in the context of

whether to start HAART in recently infected individuals. All seronegative and approximately half of seropositive detuned assay negative subjects should maintain HIV-specific immune responses by starting HAART.

3) Chapter 4 assessed the evolution of the pattern of the HIV-specific immune response in untreated PI.

   a. The individuals described in chapter 3, who maintained the breadth and magnitude of HIV-specific immune responses for at least 1 year after starting HAART early in infection, also preserved the hierarchy of this response. Subjects treated later in the first year of infection experienced an exponential decline in both the breadth and magnitude of their HIV specific effector response. In contrast, subjects in untreated PI experienced perturbations in the hierarchical pattern of their HIV specific immune response over the same 1 year follow-up period.

   b. The magnitude of the HIV specific effector response remained stable, while the breadth of the immune response fell significantly in the untreated population over 1 year of follow-up.

   c. Using variance scores (measure of the degree of variation of the magnitude of a peptide specific response at each time tested over the average magnitude of that response for the entire follow-up period) revealed that subjects in untreated PI experienced dramatic changes in the number of SFC/10^6 PBMCs responding to a particular peptide over time. Expansions appeared as variance scores greater than 1, and contractions

appeared as variance scores smaller than 1. In contrast, subjects treated early in the first year of infection, at a time where they preserved both their CD4+ and CD8+ HIV specific effector responses, experienced minimal changes in their variance scores over time.

Here, for the first time, we reported the presence of significant expansions and contractions in the HIV peptide-specific IFN-γ effector response in untreated PI.

4) Chapter 5 describes the utility of the "matrix" ELISPOT method of screening HIV specific effector responses to all expressed HIV gene products.
    a. As few as $9x10^6$ PBMCs from HIV infected patients are required to test all expressed HIV genes.
    b. Results generated in the peptide pool matrix assay and confirmed in a verification experiment identify the set of HIV peptides recognized by HIV infected individuals in an unbiased manner.
    c. In a comparison of the new matrix approach vs. the old peptide panel approach, the matrix approach was superior for the identification of the global HIV specific response.
    d. Moreover, the matrix ELISPOT identified a significant proportion of previously uncharacterized novel responses in two subjects for whom results were reported in this chapter.
        i. Twenty percent of specificities were previously undescribed for HTM 305

ii. Seven of 10 responses were novel for subject HTM 310

Here, we describe the strength of the peptide pool matrix ELISPOT approach compared to the commonly employed peptide panel ELISPOT. Application of the peptide pool matrix ELISPOT approach for screening PBMC for responses to HIV gene products will allow for the comprehensive analysis of HIV specific responses in patients at various stages of infection.

5) Chapter 6 is a comprehensive analysis of the HIV specific immune response in a group of 24 HLA-A2 HIV+ HAART naïve chronically infected subjects.

   a. Patients in chronic infection display heterogenous responses both with respect to the breadth and the magnitude of their HIV specific effector responses.

   b. Despite the fact that a trememdous amount of research has been performed on the identification of immune responses in chronic infection, as many as 37% of identified specificities within the population tested were previously unreported.

   c. Subjects in the chronic phase of HIV infection target Gag gene products predominantly.

   d. A significant proportion of the breadth was directed at the poorly characterized Vpr accessory gene product.

   e. Although subjects respond via IFN-γ release to two well-characterized HLA-A2 restricted epitopes, the majority of subjects tested are unable to mediate cytolysis upon recognition of these epitopes.

Here, for the first time, we characterized the global HIV-specific immune response pattern in a group of chronically infected patients.

Chapter 8: Discussion

8.1 Patterns of HIV specific immune activity in PI

Until recently little was known about the pattern of the immune response to HIV in early HIV PI. It is certain that the adaptive immune response at this time is very important in viral control as a critical temporal correlation has been established between the induction of CTL activity and a reduction in viral load[174]. Yet the pattern of the evolution of the immune response to the virus has been described for a limited number of patients[403-405]. Characterization of the evolution of the immune response during this critical period may reveal important clues into mechanisms of viral control. While a few studies have demonstrated that subjects in acute infection exhibit narrower responses than subjects in the chronic phase of infection[241], little work has been performed on understanding how the immune response pattern diversifies following acute infection.

In chapter 2 we explored the pattern of the immune response in treatment naïve subjects tested at different times following infection. Immune responses were monitored using a panel of HLA-restricted HIV peptides. The data revealed a number of interesting clues as to the behavior of the immune system in early PI. When PI subjects were separated into those tested within 4 months of infection and after 4 months from infection, no between-group differences were observed in the magnitude or the breadth of the HIV specific response. Yet, when the subjects tested within the first 4 months were further sub-grouped into those who were screened earlier than 2 months from infection, and those who were between 2 and 4 months from infection, a significant between-group difference in both these parameters was observed. This suggests that the immune response expands and amplifies, within the first 2 months of infection, during a critical window in the clinical course of the infection (chapter 2, Fig 2A and B).

The data presented in chapter 2 shows that in the first 2 months of infection, when most individuals are still seronegative, the adaptive immune response is developing. This window may represent the time necessary to recover from a "stunned phenotype" associated with high viremia as has been described for acute hepatitis C virus infection[406,407]. The effect of viral diversification on the evolution of an immune response can only be speculated on at this time and the data generated and presented in this thesis does not address this issue directly. The next section discusses how emerging viral diversity may impact on the evolution of HIV-specific immune responses. The cross sectional data presented in chapter 2 demonstrates that after the first two months of HIV infection, the breadth and magnitude of the immune response achieves a plateau. Longitudinal data presented in chapter 4, for untreated subjects recruited during PI, confirms that for the first year of follow up, the magnitude of HIV-specific immune responses is stable although the breadth declines and dramatic shifts with time are seen in the specificity of HIV recognition.

8.2 Why does the immune response broaden over time?

Several studies have shown that the virus from acutely infected individuals is homogeneous even when the infecting partner's virus is genetically diverse[242,408]. The transmission of a single viral species occurs more frequently in men than women[409]. Longitudinal observation of the viral sequence reveals an interesting phenomenon. It appears that the viral sequence begins to diversify as CTL responses appear[320,410-412]. There is a 4-7 day delay in the initiation of the adaptive immune response, during which time the virus can replicate relatively unaffected by antigen specific activity in the T cell compartment.

The effect of CTL activity on viral diversity was observed in patients that were treated in acute HIV infection. Altfeld et al. demonstrated that the diversification of envelope sequences began in the later stages of acute infection[241]. More simply, subjects who were effectively treated in acute infection, leading to suppression of viral load below undetectable levels, maintained homogenous Env sequences. Conversely, poor adherence in a subject, associated with several viral rebounds, led to significant changes in the viral Env sequence. This observation supports the notion that prolonged viral replication in the face of CTL activity can lead to the diversification of viral subspecies.

It has been speculated that several rounds of mutations resulting in escape from existing CTL pressure may be responsible for the generation of new epitopes that induce novel immune responses to the virus leading to a broader immune response over time. Thus narrow responses, particularly effective in suppressing viral replication, may dominate in acute infection, until viral variants emerge that can escape pressure from those few responses. As the viral variant recognized by the original dominant CTL response is overtaken by mutant virus, the size of the pool of antigen-specific clone(s) contracts allowing subdominant responses to take the forefront in the antiviral attack. With time new immunogenic peptides may be generated from new viral variants that may lead to the generation and proliferation of HIV clones to diverse gene products. Thus the correlation between the breadth and time from infection seen in chapter 2 may be a result of the production of viral variants leading to the diversification of the viral targets and vice versa.

8.3 Why is Nef preferentially recognized earliest in infection?

Several studies have presented data supporting the conclusion that the immune response in AIED is qualitatively different from that in later stages of HIV infection[241,404,413]. In addition to being narrower[241] these immune responses may be directed at different gene products at this stage of disease[321,404]. Work in the SIV infected macaque model has demonstrated enhanced targeting of accessory gene products at this early stage of disease compared to later disease stages[320,321]. Gene products expressed early in the viral life cycle, such as Tat, appear to be frequently targeted by CTLs in acute SIV infection[320,321]. Given that multiply spliced gene products dominate early in infection[49], significant quantities of this protein may be produced in early infection. Thus Tat may represent an important CTL target for the control of viral infection during the acute phase of the disease. Targets such as Tat may additionally confer added protection to the immune system as it would afford the opportunity for the removal of newly infected cells before the release of new infectious virions. To support the notion that CTL pressure on the Tat protein is an important feature of SIV acute infection, Allen et al. demonstrated the preferential incorporation of several mutations in Tat following the resolution of PI that resulted in escape from CTL pressure directed at this protein[321].

Recent studies employing strategies to screen all expressed gene products have recognized that a greater degree of targeting of accessory gene products occurs in human AIED than in chronic infection as well[404,414]. As is seen in the SIV infected macaque model, it is clear that intense targeting of accessory gene products occurs in human infection as well, suggesting that the products of early viral mRNA transcripts may be important targets of the immune response at this stage of the disease. In chapter 2 we

show that Nef derived peptides were the only ones recognized comparably in individuals tested within the first 2 months of infection compared to subjects screened at later times in PI, suggesting that immunity targeting this gene product emerged earlier than responses directed to other gene products tested. The Nef gene product is produced in large quantities from a spliced mRNA species generated early in the replicative cycle[49]. Thus substantial production of this protein early in infection may allow for the early evolution of immune responses targeting this protein. Unfortunately due to cell number constraints and the fact that accessory genes are poorly characterized, we were not able to assess the degree of targeting of accessory genes in the work presented in chapter 2. Based on knowledge described in this section it is likely that responses to other expressed accessory genes would also be detected in acute infection.

8.4 Implications for STIs?

The data presented in chapters 2 and 3 may have an important impact on several aspects of HIV patient management. Initiation of HAART in acute infection leads to rescue of HIV-specific CD4+ T cell function but also the maintenance of a restricted HIV specific response[238]. Thus it seems that initiation of therapy at this early stage of infection may prevent the development of a full and broad immune response to the virus.

Although starting treatment in acute infection may stunt the immune response, it may be critical in preventing the immunopathogenic events associated with viral replication. Thus strategies such as repeated cycles of STIs may allow for the development of novel immune responses that could eventually lead to spontaneous control of viral replication. In an effort to test this hypothesis, STIs were employed as a means of aiding in the induction of novel immune responses that could potentially control

viral replication spontaneously[238]. Subjects in acute infection were exposed to their autologous virus in order to allow for the natural induction of immune responses with repeated on/off therapy cycles. Repeated cycles would allow for exposure of the immune system to the virus, but not long enough to allow the virus to escape control.

As described in chapter 2, given that the immune response is not fully developed within the first 2 months, it may be advantageous to start HAART after the first 2 months of infection. Treatment following the first 2 months would ensure the preservation of a fully developed immune response following the natural evolution of the antiviral response. STIs administered in subjects treated following this critical immunological window may take advantage of the previously established "mature" immune response and aid in boosting additional protective responses over time. Thus the additive effects of the protective responses incurred early in infection as well as the added specificities developed with additional interventions may lead to the spontaneous control of viral infections.

On the other hand it may be that treatment in acute infection is critical for the effective use of STIs. If the breadth of the immune response evolves due to several rounds of immune escape, it may be that treatment prior to these escape events may allow for the containment of a relatively homogeneous viral species. Several rounds of short STIs may allow for short windows of viral rebound that would limit viral diversification while inducing novel immune responses. Thus the induction of several novel specificities targeting multiple areas of a homogenous viral population would make it exceedingly difficult for the virus to escape immune control. To test the hypothesis, it would be

essential to characterize the changes occurring in the viral epitope sequences during these early events of the evolution of the immune response towards the virus.

8.5 What is the effect of aggressive HAART therapy on the antiviral response over time in patients treated in PI?

While it is clear the HAART may protect immune responses induced in acute infection[238,241], little is known about the effects of HAART on the immune response when treatment is initiated later in PI. Additionally, in the context of chronic infection it is certain that these subjects experience dramatic contractions in HIV specific responses over time[283,284]. Yet several reports allude to the possibility of preserving immune responses with aggressive therapy later in PI beyond acute infection. Oxenius et al. observed subjects treated following acute infection that continued to respond to viral epitopes despite a reduction in viral load due to effective therapy[403]. Similarly, Malhotra et al. demonstrated that HAART started as late as 147 days from infection could lead to the preservation of HIV specific CD4+ T proliferative responses[319]. Thus it is clear that treatment beyond acute infection may preserve antiviral activity, yet the precise window of opportunity for this effect remains unclear. Additionally, understanding the mechanism required for the persistence of HIV specific effector responses with treatment beyond acute infection remains unresolved.

As described in chapter 3, some subjects treated well beyond acute infection preserved HIV specific responses over time. It was clear that among the subjects that were treated beyond seroconversion but during the first half of the first year of infection, 5 of 11 preserved immune responses while the remaining 6 did not. Among the 11 individuals in this group tested, all who preserved immune responses as measured by

ELISPOT assays maintained both HIV specific CD4+ and CD8+ effector responses at all times tested, while those who exhibited declining effector responses also lost both HIV specific CD4+ and CD8+ activity. Thus there was an association between the preservation of HIV-specific CD4+ T cells and the maintenance of HIV specific CD8+ activity.

Data presented in chapter 3 has implications relating to patient management. Given the difficulty in identifying patients during acute infection, the data presented in chapter 3 increases the window period during which initiation of HAART can result in preservation of antiviral immunity. Moreover, subjects treated beyond acute infection, who preserve HIV specific CD4 activity, may be equally good at controlling virus replication during therapy interruption as subjects treated in acute infection who undergo several rounds of STI to amplify HIV-specific immune responses, as mentioned above. Thus early treatment, beyond acute infection, may result in the preservation of an intact antiviral response that may support the induction of novel specificities that contribute to the spontaneous control of viral replication in such patient populations.

The information in chapter 3 is a first step to our understanding of the mechanisms behind HAART's potential effects on the persistence of antiviral activity. Intense viral replication in acute infection results in viral dissemination and cytopathic effects, leading to the infection of large numbers of CD4+ T cells[16,175]. This burst in viremia is additionally associated with intense immune activation, leading to significant immunopathogenic events. Combined, the cytophatic and immunopathogenic effects may lead to the destruction of essential immune responses. Thus it is likely that HAART, in reducing viral replication, may prevent exposure of the immune system to these

destructive events, and thus provide a means to preserve essential antiviral responses. It would be interesting to know whether individuals who maintained HIV-specific immunity among the 11 seropositive detuned negative subjects had lower peak viremia than those in whom HAART was ineffective in preserving those essential responses. Because these subjects were recruited after seroconversion this information is not available. However comparison of viral load at the last therapy naïve time point revealed no between group differences (Chapter 3 Fig 2A).

8.6 The detuned assay: a new clinical tool?

The greatest obstacle clinicians face in treating patients in acute infection is the difficulty of diagnosing infection at this stage of disease. For one, only 60-90% of subjects exhibit acute retroviral symptoms[279]. Moreover, many of the symptoms experienced during an acute infection syndrome are non-specific flu-like complaints that can often be overlooked as due to recent HIV infection[279,415,416]. The standard diagnostic test for HIV infection, a i.e a positive HIV-1 EIA, which detects antibody to Gag p24 in an ELISA, is usually negative at this early stage in infection or if positive produces an indeterminate result in a confirmatory Western blot[272,274]. Tests, such as the p24 antigen ELISA and viral load assays based on branched chain DNA or PCR can detect a presumed infection earlier than does the HIV EIA assay but high false positive results have impeded their adoption as diagnostic tests for HIV infection. Despite this, informed physicians can be alerted to possible HIV infection using these tests together with patient history information and confirm seroconversion due to infection by repeating the standard diagnostic test.

In addition to the difficulty in identifying patients in acute infection, it is equally difficult to determine the exact date of infection for a large proportion of newly diagnosed patients. Estimating an individual's exact "time from infection" can prove to be complicated as it is possible that the clinical and behavioral information may not concord. The less sensitive HIV EIA or detuned assay provides an additional objective laboratory tool that aids in assigning a time from infection. The detuned assay was developed to attempt to confirm time from infection based on the kinetics of antibody development[417]. The assay is simply a less sensitive version of the standard HIV EIA, incorporating a shorter incubation time using diluted plasma samples. Given that antibody concentration increases to a plateau over the early period of infection, subjects who produce higher optical densities (ODs) in the assay have a greater concentration of antibodies to p24 than subjects with lower ODs. Thus using data generated from a cohort of a 1000 subjects with known dates of infection, a curve was established that significantly correlates the OD with a given length of time from infection[417,418]. Thus the detuned assay scores could potentially be employed in a clinical setting to estimate the length of time elapsed since infection.

The data presented in chapters 2 and 3 can be used as the basis for clinical investigations aimed at improving patient management by increasing the likelihood of treating HIV infected subjects in PI who will derive an immunological benefit. Although current treatment recommendations include treating subjects who are in acute infection, and those with CD4 counts below 200/uL[348], the findings in chapter 3 may encourage further investigations on when and in whom to initiate therapy. The results in this chapter suggest that a significant proportion of seropositive patients who are still negative in a

detuned assay will derive immunological benefit from starting HAART. Still in question is how the balance between the long term toxicities associated with prolonged therapy, and the benefits associated with preserved immune function will play out. The best case scenario would be that preserved immune function will favor control of viral replication during therapy interruption.

8.7 What is the role of CD4 T cell help in HIV infection?

It is clear from the work presented in chapter 3 that an association exists between the preservation of HIV-specific CD4+ and CD8+ function. The precise role of CD4+ T cell help in development of functional antigen-specific CD8+ T cell responses is currently the subject of intense investigation. Several reports have found that antigen-specific CD4+ T cells are required for the development of effective memory CD8+ T cells in another chronic viral infection model (persistent LCMV infection)[214,214-217]. According to this model, CD4+ T cell help must be present during the priming phase of the CTL responses. Antigen specific CD8+ T cells induced without CD4+ T cell help respond similarly to CD8+ T cells induced with CD4+ T cell help during the primary response to virus. However, these "un-helped" CTLs display dramatic defects during the secondary response to antigen. These cells display a lethargic response pattern, exhibiting low proliferation and reduced effector activity, during the recall response[419]. It is likely that end-stage CTL activity may be compromised due to the reduced proliferative capacity of CD8 T cells that were induced in the absence of CD4 T cell help. As CTLs induced in the absence of help proliferate to a lesser extent, these cells may not be able to expand sufficiently to mount an effective CTL response. By analogy, the presence of HIV-specific CD4+ T cells may also be essential in the context of HIV infection to

guarantee the production of effective virus specific memory cells that can control the virus.

In HIV infected individuals, the virus preferentially infects naïve HIV-specific CD4+ T cells[212] making them unavailable to optimally help generate effective CD8+ memory responses. Relating this to the data presented in chapter 3, starting HAART early limits infection of HIV-specific CD4+ T cells preserving their function with respect to helping generate CD8+ T cell activity. This is perhaps what has occurred in group 1 and 2a described in chapter 3. In contrast, HIV-specific CD4+ T cell function is not maintained after HAART is started in subjects belonging to groups 2b, 3 and chronically infected persons described in chapter 3. In these individuals HIV has impaired antigen specific CD4+ T cells sufficiently so that they are no longer able to optimally help generate CD8+ responses without the presence of antigen to drive the responses. Viral load control due to introduction of HAART reduces antigen resulting in narrowing and decline in HIV-specific immune responses in these groups.

Given the high mutability of HIV, it is additionally likely that therapy, which preserves HIV specific CD4+ T cells, may allow for the induction of novel protective effector responses over time. Thus the preservation of the newly developing CD4+ T cell compartment may also be essential for the development of new responses in the context of new therapeutic strategies. Therefore, is early therapy intended to preserve both the established and novel antiviral helper responses?

In order to effectively approach this issue it is imperative to establish whether memory responses to HIV develop stochastically or if they evolve with time. Several reports support the theory that the development of memory is a stochastic event, in which

the specificity and the size of the effector pool correlates with the size and specificity of the memory pool[215,420]. Thus memory responses are pre-determined during the early events in viral infection[214-217]. The interaction between CD8+ effectors and CD4+ help appears to only be critical during the primary response, and is dispensable in the recall response to antigen[214-217]. It is possible that the early interaction between naïve CD4+ and CD8+ T cells specific for the same antigen leads to a degree of "pre-programming" which is responsible for the scheduled contraction of the immune response to that antigen. If this is the case then why aren't memory CD8+ T cells established in acute infection sufficient for controlling HIV infection?

The greatest difficulty in extrapolating these findings to HIV viral infection is that the LCMV model does not take into consideration the high mutability of HIV. Although epitopes have been shown to escape recognition in the context of influenza, LCMV and EBV[323,342,421], the majority of characterized epitopes do not appear to mutate efficiently. In the context of LCMV infection, mutations in dominant epitopes that lead to CTL evasion lead to subsequent emergence of a subdominant specificity that was also established early in infection. HIV infection may be dramatically different in that epitope evasion occurs frequently as the viral genome may be more forgiving to the error prone nature of the reverse transcriptase enzyme. As the virus changes over time, it is likely that new specificities may emerge, as the immune system is exposed to new antigens that it has never seen before. Thus the establishment of novel memory responses throughout infection may be necessary in the control of HIV unlike other persistent viral infections. It is therefore plausible that memory is an evolving process in HIV infection. Thus due to the preferential loss of HIV specific CD4+ T cells, novel responses primed later in

disease become progressively more ineffective (lethargic)[419]. Perhaps early treatment, that preserves HIV specific CD4+ T cell help, as shown in chapter 3, also leads to the maintenance of the naive CD4+ T cell compartment that may be essential for priming novel immune responses later in disease or in the context of novel therapeutic strategies such as STIs or therapeutic vaccination. It would be essential to test overlapping peptides for all gene products longitudinally in these early treated subjects in order to determine if the preservation of CD4+ T cell help consequently also leads to the creation of novel effector responses that can effectively control the virus throughout the course of the infection.

8.8 The effects of CD4 T cell loss

Gross defects have been characterized in the context of chronic HIV infection within the CD8+ T cell compartment. Champagne et al. showed that HIV specific CD8+ T cells lack a number of differentiation markers that are characteristically expressed on end stage terminally differentiated effector cells[199]. These end stage cells are essential for pathogen clearance as they possess the necessary lytic activity required for removal of virally infected cells. According to this work the functional defects seen in the CTL population in progressive disease may be accounted for by an inability of memory cells to differentiate and mature to end stage effector cells. Thus a blockade occurs in the differentiation process leading to an accumulation of cells in a pre-end stage phenotype.

Moreover, functional studies in progressive infection have elucidated the gradual loss of effector functions in the HIV-specific CD8+ T cell population such as reduced proliferative capacity, inability to lyse target cells, failure to produce IL-2, TNF-α, and finally IFN-γ[195,213]. Bourgeois et al., from studies done on murine LCMV infection,

proposed a model whereby early interactions between antigen specific CD8+ and CD4+ T cells, in collaboration with APC, lead to a DNA "programming" event, as mentioned above, where the CD8+ T cell would be able to function optimally on second encounter with antigen[215]. If CD4+ T cells are depleted, as is evident in progressive HIV infection, this signal is lost leading to ineffective priming and DNA rearrangement and thus potentially leading to the functional defects associated with HIV infection. It may be that T cell responses induced in PI, in the presence of CD4+ T cell help may be effective as they receive the proper maturation signals. As disease progresses and the virus mutates to escape these early responses, novel CTL specificities are induced with suboptimal T cell help. These CTL responses rely on the presence of high levels of antigen to drive them and would contract or disappear as viral load in controlled by HAART. As seen in chapter 3 this is indeed what happens to HIV-specific effector responses when HAART is initiated too late in PI or in chronic infection to preserve CD4+ T cell help.

A second explanation for the functional defects that are evident in the HIV-specific CD8 T cell population may be attributable to persistent viral loads[422]. In the context of LCMV infection, Wherry et al. demonstrated that while high viral loads lead to clonal exhaustion, persistent low viral loads lead to "functional exhaustion" of virus specific responses. Thus it is possible that clones that are essential in reducing viremia in PI may be clonally deleted due to high viral loads during this period. Consequently, novel clones that are induced as disease progresses may become functionally exhausted due to persistent low viral loads during the chronic period of infection.

In the murine model of persistent LCMV infection, these functional defects take effect in a hierarchical pattern beginning with a loss in CTL activity (as described in

chapter 6), followed by a loss in IL-2 secretion, then by a deficit in TNF-α secretion, ending with a loss of IFN-γ production[422,423]. Thus in addition to reduced CD4+ T cell help, persistence of low doses of antigen in HIV infection may also induce these functional defects. Yet it is possible that functional senescence, as is described here, may be a mechanism by which the immune system attempts to control the cytopathic effects induced by continual anti-viral activity, in the setting of persistent viral replication. The outcome of such a mechanism would limit lysing activity that could cause extreme tissue injury[423]. Similarly, constantly elevated levels of TNF-α can lead to high fever and septic shock, while over-production of IL-2 could lead to the uncontrolled clonal expansion of cells resulting in clonal exhaustion[422,423]. Thus persistent antigen may set off an immune mechanism that progressively shuts down activity that can be harmful to the host. These effects of persistent viral replication may be further amplified in the absence of CD4+ T cells, as in the absence of help, CD8+ T cells do not receive the signal to mount an effective response.

8.9 Lessons from untreated PI

Chapter 4 describes features of the HIV specific immune response in untreated PI. This report is the first to document changes in the pattern of the immune response over time reflecting host/virus interactions unaffected by therapeutic intervention. Longitudinal monitoring of antiviral responses in treatment naïve subjects describes the natural evolution of the host immune response to changes in viral replication. In contrast to subjects treated in early PI who exhibit a stable hierarchy in the immunodominance pattern of their HIV specific response, untreated subjects followed from PI exhibit dramatic perturbations in their HIV-specific effector responses (chapter 4, figure 1).

Given the hypothesis that several rounds of viral escape leads to the generation of the breadth of the immune response, it is possible that mutations gives rise to significant changes in the breadth of the response over time as immune responses to new specificities are induced. One of the limitations of the data presented in chapter 4 is that immune responses were monitored to peptide panels rather than all expressed HIV gene products. Despite this it was evident that dramatic changes over time were occurring in the size of the effector pool responding to particular peptides. The finding of a significant correlation between the magnitude of immune responses and the viral load (figure 4, chapter 4), supports the conclusion that changes in the level of viral replication contributes to alterations in the size of the effector pool.

Since these subjects are maintaining a viral set point, changes in the immunodominance pattern may reflect the expansion of effective responses capable of controlling replication of the dominant viral species at each time tested. From the data presented in chapter 4, we speculate that broad immune responses put pressure on the virus at multiple sites making escape of all viral variants unlikely even though escape from one specificity at a time could result in the contraction of effector cell populations specific for that variant. Fluctuations in viral quasispecies due to natural selection may lead to the elimination and the reappearance of antigen over time. A limitation of the data presented in this chapter is the absence of sequence information on the predominant viral variants present at each time immune responses were tested and information on responsiveness to variant peptides compared with the reference peptide sequences used. Generation of this type of information would elucidate the connection between virus

mutation and evolution of the HIV specific immune responses that can only be speculated on here.

8.10 Mapping the immune response to HIV:

Although it is clear that there are significant changes in the pattern of the immune response in untreated PI, it is difficult to assess the true evolution of the specificity of these responses using peptide panels targeting only previously described MHC/HIV peptide combinations. Additionally given the complex nature of the establishment of immunodominance, characterization of the changes in the breadth of the immune response is severely limited by the use of selected peptide panels as stimuli. The most significant problem with the use of peptide panels is the fact that the majority of the peptides characterized in the Los Alamos Database have been identified in the chronically infected population. Several reports have suggested that subjects in PI may have a different immunodominance pattern of HIV recognition compared with subjects in chronic infection[404,413]. Incorporating peptides from all expressed HIV genes into screening strategies has revealed responses to previously uncharacterized gene products[404,414,424-426]. Impartial assessment of responses at any stage of disease would require testing overlapping peptides derived from whole gene products. This technique would permit monitoring of changes in the immunodominance pattern via longitudinal characterization of responses. This was the impetus for the work reported in chapter 5, which explores the utility of the matrix ELISPOT.

Chapter 5 demonstrates the power of the peptide pool matrix approach for screening PBMCs from HIV infected subjects for peptide specific IFN-γ secretion.

Despite having been infected for over 6 years, HTM 310 was treated within the acute phase of infection. Chronologically one would assume that she is in chronic infection, yet the matrix analysis revealed reactivity to only 3 previously reported epitopes. The matrix screening strategy effectively identified an additional 7 uncharacterized specificities that would have been overlooked using peptide panels as stimuli. It is likely that early treatment may alter the immunodominance pattern permitting HTM 310 to recognize similar epitopes after 6 years of infection as she did in PI. It would be necessary to screen PBMC samples collected before treatment to formally show that this is the case.

Two previously unreported specificities were identified in PBMC from HTM 305, an untreated chronically infected subject. One of these novel responses mapped to the Vif accessory gene products that has been poorly characterized with respect to epitope mapping to date. Thus the data presented in chapter 5 emphasizes the advantages of using the peptide pool matrix approach over the peptide panel strategy to more fully characterize, in an unbiased manner, the HIV specific response in HIV infected patients from all different stages of infection.

8.11 Viral targets in chronic infection:

The peptide pool matrix ELISPOT described in chapter 5 was used to screen a population of chronically infected subjects in chapter 6. Despite the fact that the majority of epitopes classified in the Los Alamos Database were identified in individuals studied in chronic infection, data generated in chapter 6 demonstrated that as many as 37% of the HIV specific effector responses identified in the 24 HLA-A2 HAART naïve chronically infected subjects tested were directed at novel previously uncharacterized epitopes. Additionally, 21% of the breadth of the response was directed at epitopes within the

poorly characterized HIV accessory gene products. Thus the use of peptide panels to describe immune responses in this chronically infected population, would have underestimated the data generated with the peptide pool matrix strategy presented in chapter 6.

Using the matrix approach responses directed against Gag gene products predominated both with respect to magnitude and specificity of HIV specific responses in the untreated chronically infected population. Several studies have demonstrated that subjects in PI recognize different regions than subjects in chronic infection[241,404,413]. The chronological expression of viral proteins expressed at different stages of the HIV viral life cycle may aid in establishing why the immune response focuses on different proteins at different stages of infection, as mentioned previously. During later stages of infection, Gag, required for virion production, is highly expressed, leading to high concentrations of this gene product in the cellular environment, and thus may be more likely to be presented to the immune system[21]. Therefore it is probable that the increased concentration of structural proteins may drive the immune response to focus on these gene products probably due to the fact that the immune system cannot efficiently focus on all regions of the virus, and thus develops mechanisms to focus on gene products that may lead to the greatest level of protection.

Chapter 9: Future Directions

The work presented here has provided the background to prompt further lines of investigation.

1) Data in chapter 2 suggests that the immune response develops in the first 2 months and that treatment beyond acute infection can preserve CD4 and CD8 T cell responses (chapter 3). It would be important to address if STIs performed on patients that are treated beyond the first two months of infection, once the immune response is fully developed, will respond differently than subjects undergoing STIs after treatment in acute infection. The following research questions could be addressed:
    i. Between-group comparison of time to peak viral load rebound, level of viral load rebound, level of viral load set point off therapy and time to undetectable viremia after restarting therapy will address whether one group is better than the other at controlling viral replication off therapy.
    ii. Peptide pool matrix ELISPOT analysis will identify known or novel immune responses, associated with viral control, that are induced at the time at which spontaneous control is achieved.
    iii. Peptide pool matrix ELISPOT analyses performed before and after each STI in subjects treated at several times beyond infection may reveal that subjects treated in acute infection develop different patterns of responses with several rounds of

STIs than subjects who are treated at a time when the immune response is fully developed and HIV specific CD4 T cells are preserved.

Thus the goal of this work will be to identify novel responses associated with viral containment and to address the issue of the best time to treat to achieve the best results with STIs.

2) Longitudinal combination of the peptide pool matrix ELISPOT with viral sequence analyses can be employed to perform an in depth characterization of the evolution of the immune response during early PI with respect to changes in the viral sequence.
    i. Matrix analysis will identify previously uncharacterized epitopes that may be protective during early untreated infection.
    ii. Longitudinal characterization of immune responses following infection may reveal the preferential induction of immune responses in a hierarchical manner to specific HIV gene products over time. Moreover, changes in the immunodominance pattern of peptide-specific responses may reveal important information as to the contribution of these viral targets in the control of viremia.
    iii. Viral sequence analysis will monitor changes occurring in known and uncharacterized CTL epitopes over time during

early infection in order to link viral evolution to host immune pressure.

iv. ELISPOT analysis can assess the impact, on binding and recognition, of changes in the viral sequence within and around targeted CTL epitope variants.

Combination of the data from the two techniques, peptide pool matrix ELISPOT and viral sequencing, will describe the manner in which host and viral interactions contribute to changes that occur in the breadth and the hierarchy of the immune response following infection.

3) Finally, the matrix technique will allow for the evaluation of patterns of immune responses associated with slow progression or protection. Stratification of patients based on several clinical markers of disease progression may yield interesting patterns of immune responses associated with differential disease courses. Subjects may be stratified:

   i. By the degree of CD4 loss over time
   ii. The magnitude of peak viremia
   iii. The level of the viral load at set point

Thus, cumulatively this data will provide a greater understanding of immune mechanisms potentially associated with protection against disease progression.

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