# Evolution of the Virulent Primary Isolate, SIV/DeltaB670, *in vivo*, Implications for Study Design and Antiviral Therapies

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## ABSTRACT

Antiretroviral drug treatments and vaccine strategies are hampered by the ability of the HIV to generate variants able to evade their protective effects. Understanding the effects of these interventions on virus evolution could aid in the design of targeted antiviral strategies. We addressed this in a cohort of SIV infected non-human primates given short-term antiviral drug treatment (ART) with and without DNA vaccinations. We hypothesized that the most potent therapies (e.g. those that suppress virus burden to the greatest degree) would limit virus evolution. Our results supported this hypothesis. There was no apparent vaccine effect however. These results could indicate that the immune response was not strong enough to induce changes in the global virus population, evolution must be monitored at the epitope level to be revealed, or the most informative time points were unavailable due to low virus burdens. We additionally hypothesized that infection of the gut associated lymphoid tissue may render this organ as a reservoir for expression of unique viral genotypes. We demonstrated that high plasma virus loads were associated with high tissue virus loads and wide dissemination of genotypes. In contrast, the lymphoid tissues of animlals that controlled their virus burden contained genotypes not expressed in other organs. Our results have important implications on studying virus evolution *in vivo* by demonstrating that large populations and potentially numerous virus genes need to be analyzed.

## PREFACE

I would like to express my heartfelt appreciation and gratitude to my mentor, Dr. Mickey Corb, for her ceaseless support and encouragement during my graduate career. Dr. Corb's guidance, both emotionally and scientifically, has made my dream a reality. I will carry and use her lessons with me forever. Additionally, the members of my committee, Dr.s Barratt-Boyes, DeLuca, Flynn and Thomson have my gratitude for sharing their scientific expertise with me. Members of the "Corb lab", both past and present, unquestioningly provided me with emotional and scientific assistance. These people have become my colleagues and treasured friends. I will not mention names for fear of leaving out any one of these precious souls.

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This work is dedicated to William Harrison Massengale, you my darling, have my heart.

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# Chapter 1. Introduction

**The AIDS epidemic**

In 1981, numerous publications reported an increase in two rare diseases, Kaposi's Sarcoma and Pneumocystis carinii pneumonia, in the United States (2, 111, 137). This was the beginning of an awareness in the US of what would come to be known as acquired immunodeficiency syndrome (AIDS). The African continent had already seen the emergence of "Slim Disease" in the late 1970s. However, this was not widely known outside of the continent (187). In 1983, the virus responsible for these events was isolated (27), and had spread to Africa, Australia, Europe, North America, and South America (187). By 2005 an estimated 40 million people had been infected with the human immunodeficiency virus type 1 (HIV-1)(294), the etiologic agent that causes AIDS. This figure is 50% higher than originally projected in 1991 (294).

Sub-Saharan Africa has an estimated 24.7 million people living with HIV-1 (218). Asia has an estimated 8.3 million people infected and together: Latin, and North America with Western and Central Europe, have an estimated 3.7 million people infected with the virus. The region with the fastest growing rates of new infection varies depending on how a population is defined [e.g. geographical area (Eastern Europe, Russia, or Asia), percentage of the world population (India), or sex (Indian or African American women)], however, this demonstrates that no area or population is free from the risk of infection.

The virus has been responsible for 23.1 million deaths across the globe (295) and has left an estimated 15 million children orphaned (295). Infection and the subsequent death of those infected carries not only an emotional toll but also a financial burden on both families and governments (295) that struggle to provide succor. The spread of the virus is aided by a lack of education as to how the virus is transmitted, religious beliefs,

fear, and most urgently, civil war. In an effort to alleviate the effects of the epidemic, numerous governmental and nongovernmental organizations have formed. Their goals ranging from the eradication of the virus (International AIDS Vaccine Initiative, Bill and Melinda Gates Foundation, Global Fund, American Foundation for AIDS Research), to education (TASO, http://www.tasouganda.org/, AVERT http://www.avert.org), to caring for orphans (Nyumbani, Africare, and China AIDS Orphan Fund, Inc.). Thus far, efforts to eradicate the virus have met with little success. The reasons for this are many and varied but have to do with the virus family HIV-1 belongs to.

***HIV-1 is a member of the Retroviridae family.*** HIV-1 is in the Retroviridae family whose members can infect a wide variety of animal hosts (61). The family is divided into seven genera based on nucleotide and genome structure and includes; Avian leucosis-sarcoma virus, B-type virus, D-type virus, HTLV- BLV group, Lentivirus, Spumavirus, and Mammalian C-type virus. Infection of the host can result in in diseases ranging from completely benign (Spumavirus) to fatal infections (Lentivirus members). The members of this family share a common virion structure, genome organization, and mode of replication.

The enveloped virion is approximately 100 nm in diameter and studded with products of the envelope gene. Internal to this is the nucleocapsid (or core) that is spherical to conical in shape. Within the core are two copies of the single strand RNA genome (7 to 10 Kb in length) with the coding sequences invariably aligned *gag-pol-env* (Figure 1.1*)*. Unique to this family is a plus strand genome that does not serve as mRNA after infection but is the template used by the viral reverse transcriptase to synthesize double strand DNA (dsDNA). Integration of the dsDNA into the host's genome is

required for replication. Cellular proteins replicate the viral genome and proteins. Viral proteins are translated as unprocessed precursor proteins that assemble at the host cell membrane prior to budding. The virion is released as an immature particle that is fully infectious after the viral polyproteins are cleaved by the viral protease. HIV belongs to the lentivirus genera and the stable integration of the viral genome into the host cell is the feature that makes eradication so difficult.

***HIV-1 is in the Lentivirus genera***. Members of this genera typically do not cause disease rapidly in an infected host hence the name lentivirus (Latin: lentus, slow). Prototypical members of this genus include equine infectious anemia virus, visna virus, and caprine arthritis-encephalitis virus. Newer members include the human (HIV-1 and 2) and simian immunodeficiency viruses (SIV). Lentiviruses are considered "complex" because their genome encodes accessory proteins not found in "simple" retroviruses. These accessory proteins are often encoded by overlapping reading frames and multiply spliced mRNA. This feature maximizes the coding sequence of a small genome. The functions of the proteins vary and include aiding in transactivation, mRNA splicing, escape from immune surveillance, and viral infectivity (Figure 1.1).

***The Viral Life Cycle is divided into two phases***. HIV-1 (hereafter referred to as HIV) is transmitted as either cell free virions or cell associated integrated provirus. The replication cycle is roughly divided into an early and late phase. HIV enters cells via fusion between the viral envelope and the target cell membrane. The viral envelope spike glycoprotein, gp120, and its transmembrane anchor gp41, have critical roles in viral infectivity in the early phase. The gp120 molecule targets susceptible cells via binding to

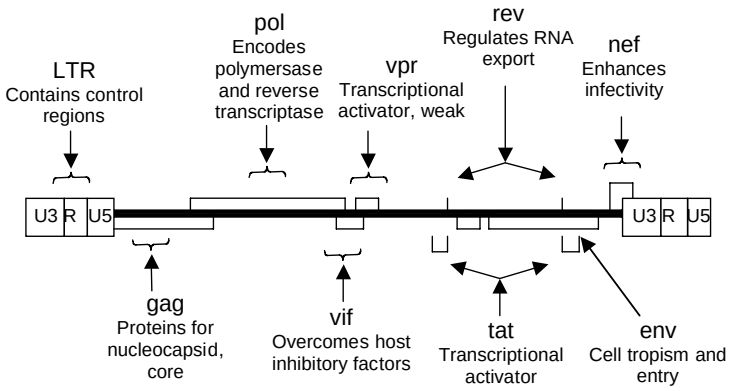


**Figure 1.1**. Organization of the HIV proviral coding regions. The genome is arranged 5' to 3' (left to right). Brief descriptions of accessory proteins are shown. [Adapted from Coffin (61), Fields Virology, Lippincott-Raven]

host cell membrane proteins. Infection after transmission is most efficient with the expression of two receptors on the target cell. The first receptor identified was the CD4 molecule (69, 152). CD4 is a member of the immunoglobulin superfamily and is expressed on CD4+ T cells, monocytes, dendritic cells, brain microglia, and macrophages. Infection and replication within CD4+ T cells and monocytes/macrophages is believed to be responsible for the majority of virus produced throughout infection (66). Binding to CD4 triggers a conformational change in gp120 that creates a binding site for another receptor. Frequently this is the chemokine (C-C motif) receptor 5 (CCR5) however other receptors are used *in vitro* (56). CCR5 was identified as a viral receptor after the CD4 molecule thus it is referred to as the co-receptor (80, 88). Binding to CCR5 triggers further conformational changes in gp120 that bring the gp41 closer to the target cell membrane and allows fusion between the two membranes. Fusion is mediated by gp41 and this allows entry of the viral core into the cellular cytoplasm where the viral nucleocapsid is partial uncoated. Within this

nucleoprotein complex the viral reverse transcriptase (RT), and host transcription factors, replicate the viral genome. Reverse transcription is initiated from a host derived $tRNA^{lys}$ at a primer binding site located downstream of the 5' LTR. Reverse transcription of the ssRNA genome is a complex process and results in degradation of the viral genome and a dsDNA copy of the viral genome. Recombination between the two RNA strands frequently occurs at this stage (see Evolution below). The nucleoprotein complex is translocated to the nucleus where the DNA is stably integrated into the host cell genome by the viral integrase protein. Integration is required for the production of progeny virions and efficient RNA transcription (278, 320).

The second phase of infection is the production of viral RNA transcripts by the host transcription machinery. mRNA synthesis is initiated at the viral LTR that contains several binding sites for cellular transcription factors. Full length transcripts serve as; genomic RNA for progeny virions, mRNA for polyproteins (gag and gag/pol), and mRNA for multiply spliced viral proteins (envelope and accessory proteins). The early phase of gene expression is characterized by multiply spliced viral mRNAs that encode nonvirion regulatory proteins that regulate viral gene expression. These include the Tat, Rev, and Nef proteins. The late phase is characterized by singly or unspliced mRNAs that encode virion proteins. Virion proteins are produced as polyproteins that assemble at the host cell plasma membrane. Virion structures, the incorporation of viral genomic RNA, and interaction with the viral envelope proteins occurs at the plasma membrane (61). Immature virions bud off the host cell membrane thus acquiring the viral envelope from the host cell. The replication cycle takes approximately 28-32 hours (149) but cleavage of the polyproteins by the viral protease is required for the production of

infectious virions (61).

***HIV Infection of Humans Eventually Leads to Death***. The initial infection (acute or primary infection) can have no attendant symptoms or can manifests itself as a flu-like syndrome (elevated fever, sore throat, nausea, malaise). During this period, the virus is widely disseminated throughout the body. The plasma virus burden is generally high (179) and an immune response is generated (Figure 1.2). The acute period can last from days to months and is often accompanied clinically with lymphopenia, thrombocytopenia, inverted CD4/CD8 ratio, and atypical lymphocytes. Seroconversion can occur from one to 10 weeks after the acute phase.

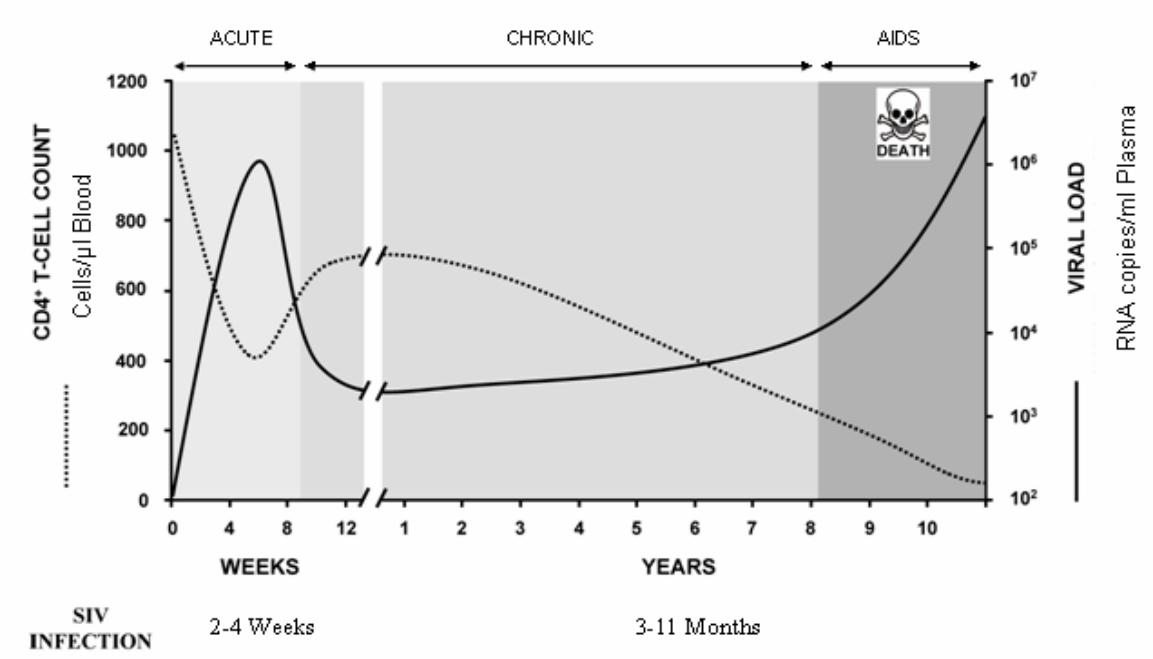


Figure 1.2. Typical course of HIV or SIV infection**.** During acute infection, viremia is coupled with a drop in CD4+ T cell counts. The immune response is initiated and virus burden declines to a setpoint, often associated with a slight recovery of CD4 T+ cells. This is followed by the chronic phase and a continuous decline in CD4 T+ cell numbers. As CD4 T+ numbers decline, opportunistic infections increase and eventually lead to death. The time differential between HIV-1 and SIV disease progression can be seen in the timeline at the bottom of the graph. [Adapted from Cohen et al. (62)].

Infected individuals then experience a clinically latent phase of infection. A decreased plasma virus load, an initial rebound or stabilization of CD4+ T cell counts, HIV specific immune responses, and an absence of clinical symptoms characterize this phase. The plasma virus load during this period has been shown to be predictive of disease progression with lower virus loads associated with longer disease free survival (197). The duration of this phase is estimated to last 10 years before the development of acquired immunodeficiency syndrome (AIDS). During this period the CD4+ T cell numbers gradually decrease. This phase is called clinical latency because no clinical symptoms are seen, however, low level virus replication is detectable in most individuals.

Gradually, the CD4+ T cell numbers decline to a point where infection caused by bacteria, fungi, viruses and parasites can not be controlled. At this point the CD4+ T cell count is very low and the virus burden is very high. The World Health Organization (WHO) uses a diagnosis of Kaposi's sarcoma, toxoplasmosis of the brain, candidiasis of the esophagus, trachea, lungs or bronchi as AIDS defining illnesses (1) because most of these conditions are controlled by a healthy immune system. The United States Centers for Disease Control and Prevention (CDC) includes HIV positive persons with a CD4+ T cell count of less than 200/microl blood in their definition of AIDS (http://www.cdc.gov/mmwr/preview/mmwrhtml/00018871.htm). With access to antiretroviral therapies the life expectancy after a diagnosis of AIDS is more than 5 years (257) however, without treatment the median survival time is 9.2 months (208).

***Virus-Specific Immunity is generated after infection***. During the acute phase of infection, both the cellular and humoral arms initiate an immune response specific to the virus.

**The cellular immune response can limit virus replication in several ways**.

The appearance of an HIV specific CD8+ cytotoxic T lymphocyte (CTL) response coincides with the decrease in the initial virus load (35, 158). CTL recognize non-self peptides when presented within the cellular major histocompatibility complex (MHC) class I molecule (315) and this recognition triggers CTL to lyse infected cells. CTL also secrete beta chemokines in an antigen specific manner, some of which (RANTES, MIP-1$\alpha$ and $\beta$) are the natural ligand for the viral co-receptor, CCR5. These chemokines can block infection through direct competition (307). CTL also secrete the Type II interferon, interferon-gamma (IFN-$\gamma$) that activates macrophages and the production of MHC molecules. It also stimulates the differentiation of CD4+ lymphocytes into T-helper 1 cells and together these responses promote antiviral activity. The importance of this response is readily illustrated by the artificial removal of these cells using the SIV infection of monkeys as model for HIV infection in humans (see SIV as a model below). SIV infected animals were depleted of their CD8+ T cells using an anti-CD8 monoclonal antibody. An increase in virus burden and in disease progression was associated with the disappearance of these cell in SIV infected macaques (142, 256). The reappearance if the CD8+ cells in the peripheral blood coincided with a decrease in the plasma virus load. Evidence is accumulating that a virus-specific CD4+ T cell response is essential for maintenance of a strong virus-specific CTL.

Matloubian et al. (193) demonstrated the importance of the CD4+ T cell response in maintaining CTL function during a chronic viral infection. Mice infected with lymphocytic choriomengitis virus (LCMV) develop strong virus-specific CTL that resolve the acute infection (6, 44, 167, 194). Using different strains of LCMV, that result

in a more persistent infection, elimination of the CD4+ T cells at the time of challenge resulted in a persistent infection of all of the mice. Importantly, these animals were unable to generate a memory CTL response to the viruses. The importance of antigen specific T helper cells in maintaining the virus-specific CTL response has also been demonstrated in chronic viral infections, cytomegalovirus and hepatitis C virus, of humans (308). HIV infection results in impaired CD4+ T helper cell function early after infection (163) and their loss as infection progresses. Although virus–specific CTL persist in the absence of virus-specific CD4+ T cells, their ability to produce IFN-γ in response to viral peptides is impaired (254). CD4+ T cells also orchestrate the humoral response to viruses.

**The Virus-specific humoral immune response**. The antibody response to HIV infection develops within weeks to months after infection (259). Antibodies to linear portions of the capsid and Gag protein develop first (260) with neutralizing antibodies mainly directed against the viral envelope protein (234) developing a variable length after infection (5, 245, 285, 310). Antibodies specific to a portion of the viral envelope protein (V3) block the interaction of the virus and its co-receptor thereby neutralizing infectivity (250). Neutralizing antibodies to the membrane spanning portion of the envelope protein (gp41) have also been identified (65). The viral envelope protein is heavily glycosylated and contains five regions of highly variable sequence that allows the virus to escape from the antibody response directed toward this protein (235, 244). Detailed studies must be performed at various stages of infection and disease to determine how best to combat infection, however, the difficulty or impossibility of performing certain manipulations in

HIV infected individuals necessitates the use of an appropriate animal model for HIV infection.

***SIV Infection of Rhesus Macaques is a Model for HIV Infection of Humans.*** HIV does not infect any nonhuman primates other than the chimpanzee (13). Chimpanzees do not reliably progress to an AIDS-like disease after infection (219, 226) and they are an endangered species so their use as an animal model is limited. In the 1980's viruses that caused an immunodeficiency syndrome were isolated from rhesus macaques (*Macaca mulatta*) housed in captive breeding colonies (30, 71, 214). Later named the simian immunodeficiency viruses (SIV) they naturally infect many species of nonhuman primates in Africa (118) with high levels of replication but no attendant disease. Experimental infection of rhesus macaques of Asian origin with tissue homogenates prepared from an asymptomatic Sooty mangabey (Cercocebus atys) of African origin (28), were shown to cause an AIDS like disease in the monkeys (28). Clinically, the animals had lymphadenopathy, splenomegaly and opportunistic infections, with most dying of enteric disease. Weight loss, thymic and lymphoid atrophy, encephalitis and syncytial cell lesions were seen. The pathology seen in these animals mirrored the pathology of HIV in humans. These results demonstrated that transmission of SIV from the natural host (sooty mangabeys) to a susceptible host (rhesus macaques) resulted in an AIDS-like disease.

SIV is 50% similar to HIV-1 and 75% similar to HIV-2, at the nucleic acid level (106). The close relationship between HIV and SIV renders SIV a suitable model with which to mimic HIV infection. The pathogenesis of SIV infection and immune response of experimentally infected macaques is very similar to that seen in HIV infected humans.

The initial drop in plasma viremia after SIV infection is associated with the CTL response (172, 256, 317), and a neutralizing antibody response is generated (42, 126). SIV isolates have been shown to mainly use the CCR5 molecule as the receptor for entry (90, 189, 296). In the animal model, the strain and dose of the virus used, the route of infection, and treatment options can all be carefully controlled.

There are three patterns of disease progression in humans and nonhuman primates that are roughly defined by the length of survival after infection. HIV infected humans that succumb to infection within 5 years are said to be "rapid progressors"while those that remain disease-free, without drug intervention, for over 10 years are called "slow" or long-term nonprogressors (174, 212). Infected individuals whose disease progression rates fall between these two extremes are called either "typical' or 'intermediate" progressors. SIV infected monkeys can also be stratified in this manner (127, 324) however, the rapid progressors typically succumb to infection within six months. The progressors succumb to infection within 1-3 years and long-term nonprogressor macaques generally remain disease free for greater than 3 years without treatment. There is also considerable interspecies variation in disease progression (286) with Chinese origin rhesus progressing more slowly than those of Indian origin. Additionally, the rapid disease progression seen in SIV infected monkeys allows *in vivo* studies to be carried out in realistic time frame.

***Virus Evolution is influenced by viral and host factors***. Both HIV and SIV exist within their respective hosts as a complex population of closely related viral variants referred to by virologists as a quasispecies. Intra-patient viral populations arise by viral evolution brought on by both viral adaptations to the host and host adaptation to the virus

(262). Viral factors include the high rate of replication, an error prone reverse transcriptase, and recombination. Studies have shown that both a large amount of virus and virally infected cells are produced and destroyed each day (128, 204, 311, 326) demonstrating the high rate of viral replication. The steady state plasma virus load is indicative of ongoing viral replication, with estimates of the average total of HIV virions produced daily of greater than $10^7$ (128, 237, 311). The viral reverse transcriptase (RT) enzyme also drives evolution. This enzyme has no proof reading mechanism therefore any errors introduced during reverse transcription of the viral genome are maintained. It has been estimated that the HIV RT introduces one mutation for every genome produced (188). Provided the mutation is not lethal, the mutated genome will be replicated. Another viral factor influencing evolution is the high rate of genomic recombination during reverse transcription (141, 173, 330). The highly variable virus population within an infected individual as well as co-infection of one cell by genetically distinct viral variants can result in the generation of virions with non-identical copies of the genomic RNA. Template switching (or copy choice) frequently occurs during the synthesis of the first DNA strand, and strand displacement during the second DNA strand synthesis both result in viral recombinants. Recombination is influenced by the integrity and secondary structure of the RNA (60, 81, 82, 103, 220). The fixation rate of mutations, i.e. the selective advantage or disadvantage of a particular mutation on the viral variant in a given environment, also influences viral evolution (59). These factors drive virus evolution and adaptation of the virus to the host.

Host factors that influence HIV/SIV evolution include the immune response generated to the virus, and intrinsic factors that can vary between individuals. The

humoral immune response induced by infection selects for viral variants that can evade antibody mediated selection pressures. Viral escape from the neutralizing antibody response is at least partially mediated by changes in N-linked (155, 245, 310) and, potentially O-linked (67), glycosylation sites within the viral envelope protein. Mutations within the envelope protein result in decreased recognition of the epitope by antibody. Evasion of the host CTL response also drives virus evolution through changes in amino acid residues that can alter T cell recognition or impair binding to the hosts MHC class I molecules (26, 31, 36, 113). The importance of viral evasion of this response is readily demonstrated by reversion of CTL epitopes after transfer to a new host lacking the MHC class I molecule to recognize the mutant epitope (100, 170). Thus another aspect of SIV infection of macaques that mimics HIV infection of humans is the escape from both the virus specific CTL and antibody response (244).

Intrinsic host factors have been identified that either prevent virus replication after entry or positively promote replication after entry. Two factors that prevent virus replication after entry are the apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G, originally named CEM15)(265) and the tripartite motif protein 5a (TRIM 5a)(280). APOBEC3G, is a member of the APOBEC family of cytidine deaminases that can deaminate cytosine on RNA and/or DNA (140). This results in G to A hypermutations in the viral cDNA (166, 186, 323) and a loss of integrity and stability of the viral cDNA and its ability to encode functional proteins (122). TRIM 5a is a cytoplasmic protein that restricts infection of a variety of viruses. The mechanism whereby TRIM 5a exerts this effect is currently unknown, however it was known that mutations to the viral capsid protein that change the stability of its association with the

viral core affect reverse transcription (99). This suggests that a cellular process that changes the kinetic of uncoating might also affect reverse transcription. Both of these proteins act in a species-specific manner so their development as antiretroviral agents await further research into their mechanisms of action. Attempts to identify other cellular restriction factors are underway (277).

The host cellular factors that oppose virus replication are joined by cellular factors that promote the spread of the virus. Two of these factors are emerin and the cellular tumor susceptibility gene 101 (Tsg101). To support a productive infection, the viral cDNA must be integrated into the host cell genome. This is accomplished with help from the inner-nuclear-envelope protein emerin (138). In cells lacking emerin the viral cDNA enters the nucleus but integration is inefficient. Other cellular proteins are also required for the viral cDNA to interact with emerin [barrier-to-autointegration factor and LEM (LAP,emerin,MAN)]. For the virus to spread within an infected individual efficient budding must occur and this is accomplished in cooperation with the cellular Tsg101 (107) in concert with another host protein of the vacuolar protein sorting machinery, AIP1 (279).

The hosts MHC haplotype as well as genetic polymorphisms can influence their susceptibility to infection or disease progression. Expression of particular MHC alleles have been associated with either rapid or slow disease progression rates in humans or macaques (34, 47, 182, 202, 230, 282). Single nucleotide polymorphisms in the promoter region of the cytokine IL10 gene have been associated with both an increased risk of HIV infection and faster disease progression with lower CD4+ T cell counts (268). Additionally, individuals who are heterozygous for a 32 base pair deletion in the CCR5

gene, the viral coreceptor, have been shown to have slower disease progression rates after infection. Individuals who are homozygous are resistant to HIV infection (73, 133, 198, 201). Together, intrinsic host factors that oppose or promote the spread of the virus can have a large impact on disease progression. Currently, the only treatment for HIV infection is by chemotherapeutic agents that target different viral proteins/enzymes.

***Antiretroviral Drug Therapy is currently the only available treatment for infection.*** Numerous points in the virus lifecycle provide targets for antiretroviral (ARV) agents. The advent of antiretroviral therapy (ART) was heralded by the introduction of 3'-azido-2',3'-dideoxythymidine (AZT, commonly called zidovudine) in 1987 (96). AZT blocks productive infection of new cells by preventing replication of the viral genome prior to its integration into host cell DNA. This drug belongs to a family of drugs called nucleoside analogue reverse transcriptase inhibitors (NRTI). AZT proved efficacious in lowering the plasma virus burden, however, mutations in the viral RT result in virions with reduced sensitivity to the drug (97, 105, 164, 274). Since the introduction of AZT, drugs that target other viral proteins/enzymes have been introduced.

Fusion inhibitors bind the viral gp41 and inhibit the conformational change needed for gp41 to mediate fusion between the viral and cellular membrane thus blocking the viral core from entering the host cell. Protease inhibitors (PI) bind the active site of the viral protease and prevent processing of the viral proteins necessary for production of infectious progeny. The nonnucleoside RT inhibitors (NNRTI) bind the viral RT itself and prevent replication of the viral genome. With FDA approval of new ARVs, it was reasoned that drugs used in combination would prevent the accumulation of drug resistant

mutants because more mutations would be necessary to evade all the drugs ensure viability.

In 1996 highly active antiretroviral therapy (HAART) was introduced (4) and typically consists of a combination of three or four antiviral agents. Studies have clearly shown that HAART extends both the disease free survival and life expectancy of HIV infected individuals (83, 91, 233). However, treatment does not eliminate infection so viral recrudescence (55), and its attendant risks (20, 64) is a constant danger. Unfortunately, soon after HAART was introduced it was determined that replication competent virus with reduced sensitivity to all drugs were found, and transmitted (283, 291). The macaque model of HIV infection has proved useful in identifying viral reservoirs while on therapy (266), the effects of treatment on CD8+ T cell subsets (319) and viral rebound after treatment withdrawal (225). This model has also demonstrated that potent combinations of ARVs can limit viral rebound after therapy is withdrawn (125) thereby limiting the evaluation of immune interventions delivered during drug treatment.

Numerous ARVs have been tested in SIV infected non-human primates, however, [also known as (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA)] (Gilead Sciences, CA) has been the most widely used. PMPA is an NRTI that was originally shown to protect non-human primates from SIV infection when administered from 48 hour before to 24 hours after SIV infection (290). These results were not consistently reproducible (125, 143), however, PMPA can be effective in lowering the plasma virus burden during treatment but it does not always limit viral rebound after its withdrawal. As seen with other NRTIs, mutations in the viral RT (K65R) results in resistance to

PMPA in both HIV and SIV (116, 298, 322). This mutation confers a discrimination-resistance in which the viral RT has reduced affinity for the drug when compared to the natural dNTP substrate (270) and also confers resistance to other NRTIs. This mutation results in increased processivity of the HIV RT during DNA synthesis (17), indicating that the fitness of the mutant virus may not be altered in the presence of drug. The constantly evolving viral population limits the effectiveness of both ART and the immune response to infection, so strategies are needed to curtail the rate of virus replication, thereby limiting viral evolution and escape from the immune response.

***DNA Vaccines show promise in limiting disease progression after infection.*** Historically, vaccines for infectious diseases have the greatest impact on public health. With respect to HIV, the belief that the immune system can control virus replication and/or infection comes from multiply exposed individuals who remain virus negative (248) and long term infected individuals who remain clinically healthy (nonprogressors) (57, 267, 321). The goal of a vaccine is to produce a memory immune response so that subsequent exposure to the pathogen results its rapid and effective clearance from the host, or saves the host from the deleterious effects of infection. Exogenous antigen, presented via the vaccine, may either boost the existing immune response or generate a *de novo* immune response different than that induced by natural infection. Therapeutic vaccination assumes that enhancing or generating anti-HIV immune responses can modify the clinical disease course. A number of different vaccine preparations have been tested against HIV using the SIV model (58, 132, 191, 213, 316). Thus far the preparations have not proven safe for use in humans or provided sterilizing protection from infection.

A newer vaccine technique currently being developed is the DNA or genetic vaccine. DNA vaccines code for a protein antigen(s) of choice under the control of a mammalian promoter. The DNA is amplified for mass production as a bacterial plasmid and delivered into the recipient parenterially (intramuscular or intradermal), or directly into cells using a particle mediated delivery (PMD) device or gene gun. The advantages of this method include the speed of production, inexpensive production costs (as compared to protein based), and the stability of the DNA at ambient temperatures, thus obviating the need for refrigeration. Additionally, multiple genes can be incorporated into the DNA vector, to engineer the immune response. This vaccine technology has been shown to elicit both antigen-specific humoral and cell mediated immune responses to a wide range of diseases and infectious agents (159).

DNA vaccination relies on the uptake or delivery of the DNA into the cell's nucleus and its subsequent transcription and translation into proteins. Using gene gun technology, the DNA is deposited intracellularly (92, 314), circumventing the necessity for uptake. This approach mimics aspects of vaccination with a live attenuated vaccine without the safety concerns associated with an infectious agent. The endogenous production of antigen results in a protein with native posttranslational modification, conformation, and oligomerization. This was demonstrated by the induction of anti-haemagglutinin (HA) antibodies in nonhuman primates vaccinated with DNA encoding the influenza virus HA (292), that is only efficiently transported to the cell surface as a trimer.

Gene gun technology can utilize the skin for targeted delivery of DNA vaccines, so called particle-mediated epidermal delivery (PMED). The use of the skin for

induction of an immune response has proven to induce both type 1 T helper cell responses, and robust cytotoxic T cell responses to HIV and SIV (101, 120, 131, 147). Transfection of both keratinocytes and resident dendritic cells (DCs) (63, 239) results in the production of the encoded proteins. The skin has resident epidermal and dermal dendritic dells (DCs) in a resting state. In response to danger signals the DCs will become activated and upregulate MHC class I and II molecules in addition to costimulatory molecules (3). DCs will also begin to express the CCR7 molecule, the receptor to the secondary lymphoid tissue chemokine, for migration to the draining lymph node (86). DCs can present antigen loaded onto either the MHC class I or class II molecules (94, 253, 318) within the lymph node to stimulate antigen specific naïve T cells or primed T lymphocytes to proliferate and differentiate.

Concerns regarding this technique include the possibility of recombination between cellular DNA and the vector, and the possible immunogenicity of the DNA itself. The fear is that recombination could result in the incorporation of a strong promoter before an oncogene, or inactivation of a tumor suppressor gene. No integration of the vaccine DNA into the host genome was detected in one study specifically researching this possibility. The authors calculated that the frequency of recombination would be less than that expected by spontaneous mutation alone (221). Another fear is that a lupus-like autoimmune disease could develop due to the possible immunogenicity of the DNA itself. One study in mice demonstrated that parenteral delivery of a DNA vaccine did not alter the disease course in lupus-prone mice (207). However, optimization of a peptide dsDNA mimetope induced antibodies that were frequently cross reactive with DNA (29). Whether DNA vaccines will induce anti-DNA antibodies in humans is

currently unknown, however, based on preclinical animal studies this seems unlikely. DNA vaccines have provided protection from disease progression (25) in macaque models of HIV infection. Challenge studies using homologous, pathogenic or avirulent SIV viruses have shown that this strategy can provide significant protection from disease progression (25, 171, 246). This technology has also proven to induce a protective effect against a heterologous, virulent isolate in the mucosal SIV challenge model (101). Additionally, the proven ability of a DNA vaccine to prevent a disseminated infection after a mucosal challenge (101) argues that this technique may be able to limit the establishment of a viral reservoir, one major obstacle to eradicating the virus.

***Viral Reservoirs make eradication difficult.*** A viral reservoir could be an infected cell in a quiescent state or a virion that has not yet infected a cell. Under drug therapy, the reservoir could be a drug privileged site or one with poor drug penetration. Antiretroviral therapy (ART) does not eliminate all virus replication or the viral reservoir of infected cells. A quantative plasma virus load in some patients while taking ARTs, or viral rebound when the drug is removed readily illustrates the presence of a reservoir. Additionally, the demonstration of continued virus evolution indicates; the drug is not 100% effective, there is incomplete drug penetration, or other viral reservoirs exist that allow virus replication under treatment. Studies have shown the memory CD4+ cell population is a cell population containing integrated proviral DNA (54, 95) that actively replicates infectious virions upon stimulation, (55, 313). Additionally, B cells (139, 185) and dendritic cells (DC)(43) have been shown to traffic infectious HIV virions. The DCs have been demonstrated to retain virions in an infectious state for a minimum of six months (43). It was recently demonstrated that in some infected individuals the source

for plasma virus was the blood monocytes (328). The viral reservoir contains a mixture of genotypes representing both ancestral and modern variants (224). The ancestral variants present before the initiation of antiretroviral drug therapy may be more virulent than the drug resistant variants circulating during treatment. Even in patients experiencing treatment failure, continuing antiretroviral therapy may suppress this more virulent reservoir.

The number of people infected with HIV is estimated to be greater than 40 million, with 11,000 new infections occurring daily. The current standard of care is antiretroviral drug therapy. This treatment has proven to prolong the disease free survival after infection however, the majority of those infected do not have access (136, 293) or are even aware of (51) its benefits. Additionally, not everyone responds well to treatment and even if the response is initially favorable, mutant genomes with reduced sensitivity to drug arise and bring a return of disease progression and thus far, inevitable death. An effectively strategy is needed to halt the spread of the virus both within and between the worlds population. One obstacle to eradication is the ability of the virus to rapidly mutate thus escaping the effects of both the host's immune response and antiretroviral drug therapy. Understanding the effects of drug treatment and/or vaccination on virus evolution *in vivo* is one step towards designing strategies to halt this growing pandemic and allowing those infected with HIV to die of old age.

# Chapter 2. Specific Aims

Numerous HIV infected individuals are currently prescribed antiretroviral therapy (ART) to aid in controlling viral replication, with the goal of prolonging their disease free survival. Several HIV vaccine trials are also underway (7) with the same goal. Viral evolution is influenced by many factors including the selective pressures of the host's immune response, drugs, and/or vaccine and the adaptation of the virus to its host. The impact of these factors on viral evolution and response to antiviral therapies *in vivo* is largely unknown. Additionally, preferential infection and elimination of the CD4+ cell population within the gut associated lymphoid tissue (GALT) occurs during acute infection. The effects of these events on the amount of virus present in the GALT and the viral variants expressed in these tissues are largely unknown. This understanding is important in the design of future antiviral interventions.

Using the SIV infection of macaques as a model for HIV infection in humans we proposed to analyze the effects of drug treatment with and without vaccination on virus evolution in SIV infected macaques. We also proposed to determine the amount of virus contained in the GALT and if preferential expression of genotypes occurred in this tissue after an established infection. Our hypothesis is that **animals that respond favorably to ART and receive a vaccine preparation targeting defined CTL epitopes will display less viral evolution than animals that responded poorly to therapy and received a vaccine preparation targeting only T helper responses. We additionally hypothesized that the GALT would contain genotypes unique to the site and contain more viral copies than peripheral lymph tissue.**

To test these hypotheses the following specific aims were constructed.

**Specific Aim 1. Determine the presence and extent of quasispecies evolution in responder and nonresponder animals using heteroduplex tracking analysis on longitudinally sampled plasma virus cDNA to determine if a correlation exists between epitope recognition and V1-V2 variation.** The goal of this aim was to monitor the evolution of viral genotypes in SIV/DeltaB670 infected animals. To accomplish this goal we optimized the heteroduplex tracking analysis (HTA) for use with our isolate, SIV/DeltaB670. HTA was then performed on longitudinally sampled cDNA from forty-four SIV infected animals; left untreated, given transient drug treatment, given DNA immunizations during transient drug treatment, or primed with a DNA vaccine and boosted with the DNA immunizations during transient drug treatment. The HTA patterns representing the expressed viral genotypes were compared to determine the effect of antiviral therapies on virus evolution.

**Specific Aim 2. Determine whether the mesenteric lymph nodes harbor unique genotypes and/or replicates more virus than axillary or inguinal lymph nodes.** Using real time RT-PCR we quantitated the viral copy number from mesenteric, axillary, and inguinal lymph nodes as well as jejunum harvested at necropsy from animals that had been infected a minimum of 11 months. The viral copy number was quantitated and cDNA was prepared from total RNA isolated from these tissues. The copy number was normalized to the expression levels of β-GUS and the results were stratified according to the plasma virus burden to compare the virus loads between tissues. HTA was performed on the tissue derived cDNA and the results were compared

among the tissue as well as the virus circulating in plasma at necropsy to determine the genotypic expression patterns.

# Chapter 3. Development of the Heteroduplex Tracking Analysis (HTA) to Monitor the Evolution of SIV/DeltaB670.

***Introduction.***

HIV/SIV exist within an infected host as a complex mixture of closely related genotypes referred to as a quasispecies. Genetic variation arises from both host (25, 34, 36, 113, 155, 245, 310) and viral factors (128, 141, 173, 188, 311, 330) and can be associated with viral fitness (59). The ability to monitor viral evolution within an infected host allows us to monitor the development of drug resistance (105, 164, 283, 291, 298), study viral pathogenicity (19, 50, 203, 252, 286), and track the epidemic (78, 105, 157). Heretofore this has been a time consuming and costly process, based on amplification and sequencing of viral genes. These limitations restrict the number of samples that can be analyzed so informative time points, or samples from a large population may be missed. Our laboratory's primary focus is SIV vaccine research and an understanding of the effects of the vaccine on virus evolution *in vivo* is imperative to designing an efficacious vaccine. We chose to develop the heteroduplex tracking analysis (HTA) for use with our primary SIV isolate, DeltaB670. HTA is a method that can monitor evolution within a chosen region of a genome by identifying mutants based on the differential migration of duplex DNA (76). This analysis would allow us to rapidly monitor and/or select specific mutants for further study.

For this analysis, a region of interest is selected and amplified from both a reference genome and unknown samples. The region must be flanked by constant sequences to allow for reliable amplification from the unknown sample as well as allow for an informative analysis. The reference sequence (probe) is amplified as a single strand and labeled to enable its detection. The probe and unknown are mixed to allow for duplex DNA formation and the dsDNA is then separated through a highly resolving gel

matrix. Insertions/deletions or base pair changes between the two strands retard the migration of the dsDNA through the gel and result in reproducible banding patterns (23). We chose to monitor changes within the V1-V2 region of the viral envelope gene because this has been used to identify SIV/DeltaB670 genotypes (287) and the envelope protein is a primary target of the host's immune response.

Due to its external location on the virion, envelope is an antibody target (235, 244, 250) and studies mapping viral epitopes have also identified CTL targets within this protein (12). The envelope protein is heavily glycosylated and dictates the cell type the virus can enter through its recognition of the viral receptor (CD4) and co-receptor (frequently CCR5 or CXCR4). Studies have shown that changes in the variable regions V1, V2 and V3 can potentially affect the pathogenicity of the virus by acquiring the ability to enter new cell types (50). Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels (50). Viral escape from the host's CTL response has been demonstrated in HIV infected humans (36, 144) and SIV infected macaques (228). These findings suggest that the ability to track sequences within the envelope gene throughout infection may lend insight into the identification of genotypes associated with enhanced fitness and the development of efficacious vaccines.

SIV/DeltaB670 is a primary isolate that consists of a complex mixture of over 20 genotypes that can be identified by distinct sequences in the V1-V2 hypervariable region of gp120 (16, 286). We thus chose to use this region to track specific genotypes during the virus evolution associated with antiretroviral and vaccine therapy and/or disease

progression. Although studies have suggested that alterations in glycosylation within V1-V2 may enable the virus to escape antibody recognition (238, 249), this region has not thus far been shown to encode T cell epitopes. Mutations elsewhere in gp120, outside of the V1-V2 region do encode T cell epitopes, however, with mutations at these sites leading to escape from CTL recognition (48, 144, 272). Additionally, this portion of the genome is the most hypervariable region of the genome (231) and should therefore provide a sensitive indicator of viral evolution.

To develop a rapid method for examining virus evolution in a large number of samples we chose to develop the heteroduplex tracking analysis (HTA) to monitor changes in the V1-V2 region of envelope of SIV/DeltaB670. This chapter describes the parameters of HTA for the SIV system studied here.

*Materials and Methods*

**V1-V2 *env* PCR amplification and probe generation**. To determine the V1-V2 sequence to use as the probe for the HTA, this portion of envelope gene was amplified in independent duplicate, PCR reactions containing 1 ng of DNA from three separate plasmids containing either the SIV/DeltaB670 clones 3 and 12, or SIV/17E-Fr envelope region by nested PCR as described (287). This resulted in the generation of non-specific PCR products as demonstrated by the presence of additional band on ethidium-bromide stained agarose gels. We repeated the amplification using only the second round primers and this resulted in amplification of one fragment of the correct size (~480 bp). The amplicons were gel purified using a commercially available kit, per manufacturers instructions (Promega, Madison, WI). 10 ng of these amplicons were then amplified and radiolabeled by asymmetric PCR using the second round forward primer as described (281). This product was gel purified (Qiagen, Valencia, CA) and stored at –20 C before use.

**Heteroduplex tracking analysis (HTA).** Nucleotide changes in the V1-V2 region of *env* were monitored by HTA. HTA was performed using the following modifications of the procedure originally described by Delwart and Gordon (76). The probe amplicons were pooled, gel purified (QIAEX II gel extraction kit, Qiagen, Valencia, CA) and eluted in 50 μL water. Amplification and gel purification was repeated using 1 ng of the gel purified product as starting material.

The radiolabeled probe was generated by asymmetric amplification of 10 ng of the gel purified product in a 100 μL reaction using only the second round forward primer. The cycling conditions were: 1 X PCR buffer II, 50 mM $MgCl_2$ 0.75 mM dCTP, 0.75

mM dTTP, 0.75 mM dGTP, 1:6 diluted dATP, 10 μCi $^{32P}$ dATP, 2.5 U Amplitaq gold. The resulting radiolabeled single strand probe was subjected to electrophoresis at 60 mV for approximately 3 hours, visualized by autoradiography, excised, gel purified, and stored at –20 °C until use.

Fifteen μl of pooled V1-V2 PCR products were individually mixed with 5 μl of a master mix containing 1 μl of radiolabeled single strand probe, 2 μl HTA buffer (1 M NaCl, 100 mM Tris, pH 7.4, 20 mM EDTA) and 2 μl water. The mixture was heated to 93 °C for 3 min and immediately placed on ice. The duplexed DNA was subjected to electrophoresis in 5% MDE (Cambrex Bio Science, Rockland, ME) in 1X TBE buffer (1 M NaCl2, 100 mM Tris pH 7.5, 20 mM EDTA) at 300 V for 3.5h. The gel was dried and the bands were visualized by autoradiography.

**Dilutions for HTA detection limit**. The optical density of plasmid preparations was used to determine the number of envelope copies in each preparation. The plasmids were diluted to the desired copy number, then mixed before PCR amplification. The V1-V2 region was amplified using nested PCR as described (287) with independent reactions containing $10^4$ through $10^2$ copies of the envelope region. Reactions were performed in duplicate or triplicate. 10 μl of each reaction was used for HTA.

***Results***

*Study design.* To develop a rapid method to monitor viral evolution *in vivo* we optimized the heteroduplex tracking analysis (HTA) for use with our primary isolate, SIV/DeltaB670. We optimized the analysis using the V1-V2 region of the envelope gene amplified from plasmids containing this region from individual SIV/DeltaB670 clones. Briefly, Trichel et al. (287) had prepared these clones using PBMC DNA isolated 2 weeks pi (post inoculation) from animals that had been inoculated either intracolonically, orally, or intravaginally with SIV/DeltaB670. We chose to optimize the HTA using these samples because their sequence was known and they represented genotypes in which a minimum of virus evolution had occurred. The use of these plasmids also eliminated the inherent danger of working with infectious virus.

*SIV/DeltaB670 Clone 3 was selected as the genotype to monitor throughout infection.* SIV/DeltaB670 consists of a minimum of 20 genotypes as determined by sequencing the V1-V2 region of the envelope gene (16, 287). To determine which clone to track we selected SIV/DeltaB670 clones 3 and 12, and the isolate, SIV-17E. Clones 3 and 12 are two of the dominant genotypes in an inoculum and share approximately 87% identity at the nucleotide level to the other DeltaB670. SIV-17E was selected because it is approximately 25% identical to the DeltaB670 stock and we wanted to determine if this greater diversity would provide greater discrimination between the DeltaB670 genotypes (Figure 3.1). We performed HTA using each of these three genotypes to probe the V1-V2 regions from the other clones. This analysis demonstrated that the greater percentage of mismatches between 17E-Fr and the DeltaB670 genotypes did not result in a clear distinction between the two DeltaB670 genotypes (Figure 3.2). We also identified an

```
B670-3    TGGGGGTTAA CAGGGAATGT A...CCAACA ACAACAGCAA CAACA..... ....TCAACA ACAACACCAA AAGAAA...C
B670-12   ---------- ---------C -...G----- ------A--- -----ACAAC AGCA------ ---------- ---G--GAG-
B670-9    ---------- ---------- -...A----- ---------C -----ACA.. .GCAA----- ---C--AA-G ------...G-
B670-22   ---------- ---------C -...G----- ---------G --T--GTA.. .ACA------ G--G------ ---.......
B670-6    ---------- ---------C -...A----- ---------- -----GCAGC ACCA------ -G-------- ---T--CAG-
B670-10   ---------- --A------- -GAAA----- ------A--- -----ACA.. .ACA------ ---------- ---......T
17E       -----A--G- --A..---CA -....T---- ---------T -----ACA.. ....-----G ---G--T--G C-AG-...GT

B670-3    AAATGTTGTA AATGAAACTA GTTCTTGTGT AAAAAACAAT AATTGTACAG GCTTAGAGCC GGAACCA
B670-12   -G-------- ---------- ---------- ---------- ---------- ---------A A------
B670-9    -G-------- ---------- ---------- ---------- ---------- ---------A -------
B670-22   .....----- ---------- ---------- ---------- ---------- ---------- -------
B670-6    ---------- ---------- ---------- ---------- ---------- ---------A -------
B670-10   -G-AA----- ---------- ---------- ---------- ---------- ---------- -------
17E       -G-CA-G--C -----G---- --------A- -GCCC-GG-- -----CG--- ----G--A-A A--G-A-
```

Figure 3.1. Sequence alignment of the V1 region of the envelope gene from six SIV/DeltaB670 clones and SIV-17E. Sequences were aligned to SIV/DeltaB670 clone 3, top line, with identical nucleotides replaced with dashes and insertions represented by dots. The SIV/DeltaB670 clones are indicated by B670 followed by clone number.

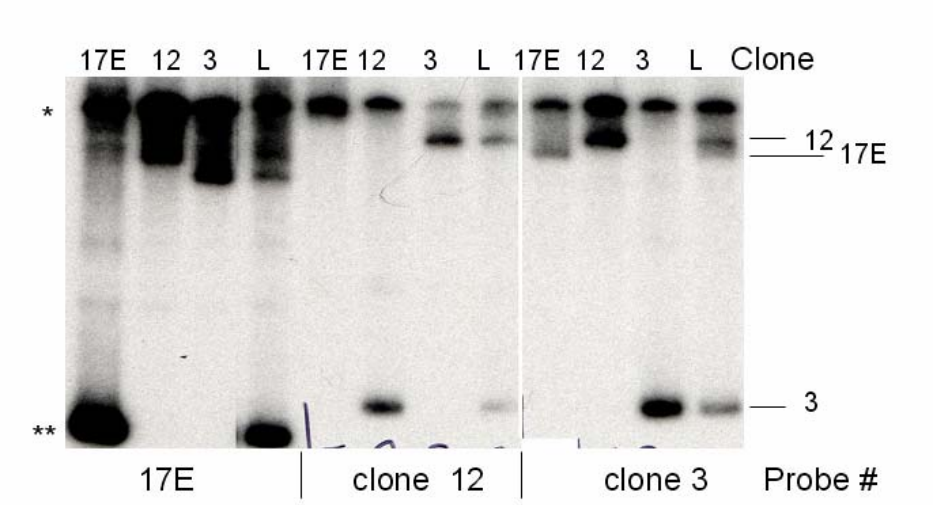


Figure 3.2. HTA demonstrating the migration of SIV-17E and SIV/DeltaB670 clones 3 and 12 probed with the same clones. Probes were generated from the indicated clones and individually mixed with each individual clone alone. The clones are indicated across the top of the figure. L contains a mixture of the clones. The asterisk and double asterisk indicate the single strand probe and homoduplex position respectively.

additional band in the lane containing only the 17E-Fr isolate demonstrating the PCR conditions were not optimal for this sample. Probing these genotypes with DeltaB670 clone 12 resulted in a reduced ability to distinguish 17E-Fr from the single strand probe lane (Figure 3.2, 5$^{th}$ lane). This outcome could preclude our ability to distinguish DeltaB670 genotypes with the same degree of identity to the 17E genotype. The use of the DeltaB670 clone 3 resulted in a separation between these genotypes present so this clone was chosen for further analysis.

*SIV/DeltaB670 clones have unique migration patterns*. To determine the migration patterns of the other DeltaB670 genotypes by HTA, we chose to compare SIV17E and the DeltaB670 clone 3. We reasoned that although there was less identity between DeltaB670 genotypes and 17E, this might provide greater distinction between the genotypes than that provided by DeltaB670 clone 3. The results, shown in Figure 3.3, demonstrate that the greater degree of sequence identity between the DeltaB670 clone 3 and the other genotypes resulted in greater separation between the genotypes on the gel. This factor would allow for greater discrimination between genotypes when more than one variant was present within a sample, so we selected DeltaB670 clone 3 for further development.

*A clone representing greater than 10% of the sample is reliably detected by HTA.* To determine what percentage of the sample one genotype would have to represent for accurate detection by HTA, we performed a dilution experiment in which one clone represented from 50% to 1% of the mixture. We used clones 3 and 12 for this experiment, holding the concentration of clone 3 constant. We amplified the V1-V2 region of the envelope gene in independent reaction containing a serially diluted clone 12

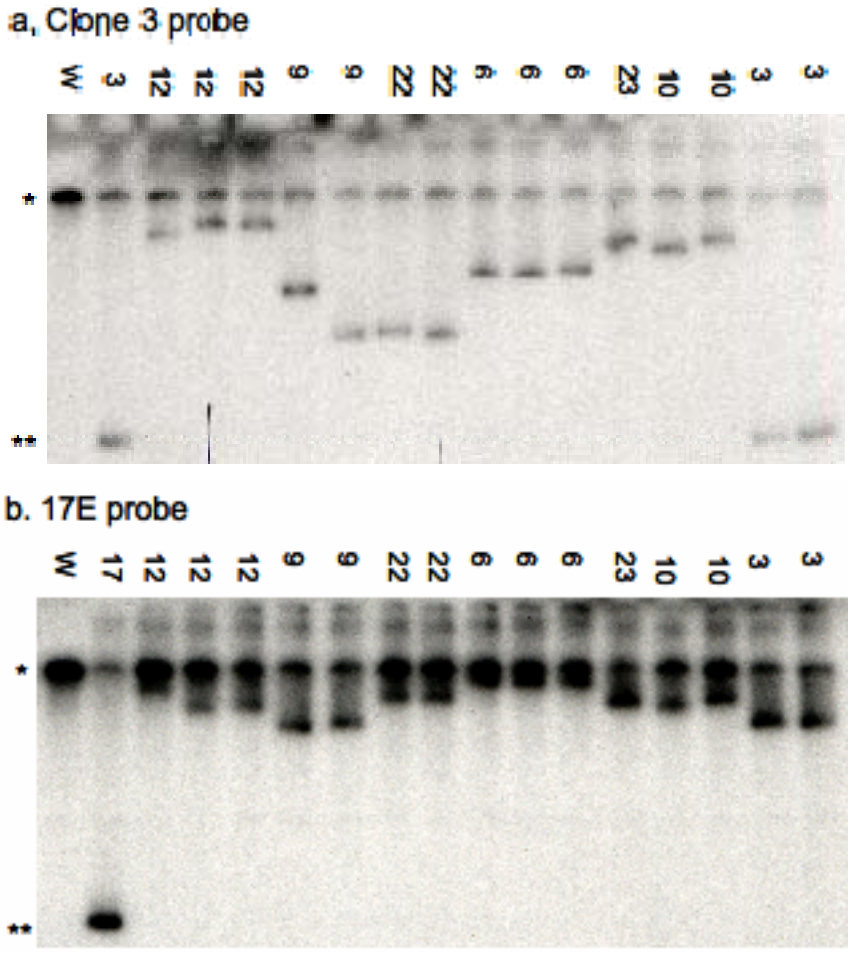


Figure 3.3. HTA demonstrating the migration of SIV/DeltaB670 clones tracked with SIV/DeltaB670 clone 3 (a) and SIV-17E probes (b). Probes were generated from SIV/DeltaB670 clone 3 and SIV-17E and mixed with individual SIV/DeltaB670 clones. The V1-V2 region was amplified from plasmids containing a portion of the envelope gene from the clones indicated across the tops of the figures. W indicates the probe mixed with water alone. The asterisk and double asterisk indicate the migration of the single strand probe and homoduplex position respectively.

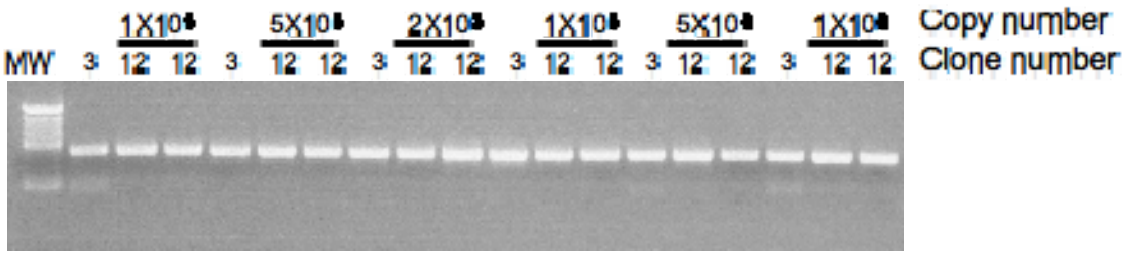


Figure 3.4. Ethidium bromide stained agarose gel containing amplicons from SIV/DeltaB670 clones 3 and 12. The template copy number and clone number are indicated across the top of the gel. The template copy number was calculated based on the optical density of the plasmid preparation. The copy number of clone 3 was held constant at $1X10^4$ and mixed in duplicate with dilutions of clone 12 before PCR. MW represents the molecular weight marker. The negative controls are not shown as no PCR product was generated.

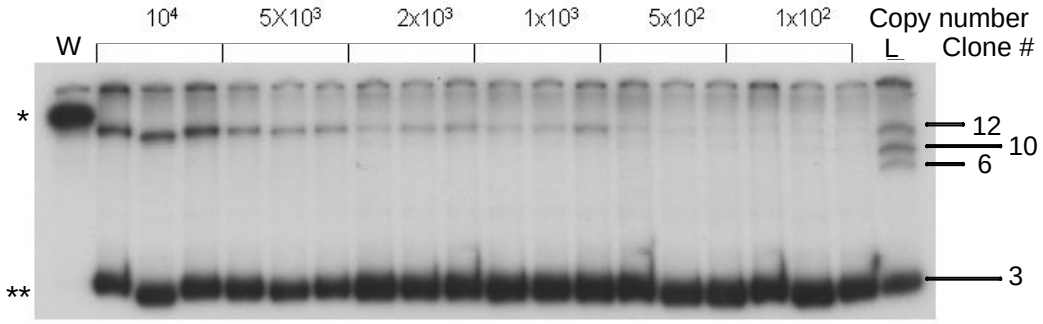


Figure 3.5. HTA demonstrating the detection limit of one clone. Plasmids containing SIV/DeltaB670 clones 3 and 12 were to the desired concentration based on the optical density of the plasmid preparation. The concentration of clone 3 was held constant at $10^4$ and mixed with clone 12 that was serially diluted to represent from 50% to 1% of the mixture, indicated as copy number at the top of the figure. W is the probe with water alone, L is a clone ladder with the individual clones identified on the right. The asterisk and double asterisk represent the single stand and homoduplex positions respectively.

and 1 X $10^4$ copies of clone 3. We were able to reliably amplify 1 X $10^2$ copies as judged by ethidium bromide staining of an agarose gel (Figure 3.4). HTA revealed that we could reliably detect the variant that represented 10% or greater of the input DNA (Figure 3.5).

We repeated this experiment using six SIV/DeltaB670 clones (12, 9, 6, 22, 23, and 3) to determine if this level of detection was possible with a more complex mixture of genotypes. Clone 6 was diluted to represent 9% and 4.5 % of the HTA reaction mixture. We were not able to reliably detect clone 6 when it represented 4.5% of the mixture (Figure 3.6) therefore we determined a clone had represent at least 10% of a sample for reliable detection.

*A plasma virus copy number above $10^3$ is required for reliable HTA results*. To determine the plasma virus copy number a genotypes must represent for reliable detection by HTA we performed serial dilutions of four different Delta/B670 clones to mimic different copy numbers *in vivo*. We demonstrated by HTA that reproducible results were obtained when the copy number was greater than 1 X $10^3$. At this copy number the banding patterns of the genotypes were not identical (Figure 3.7). This result could be due to the sequence identity between the probe and the inclusion of its identical homologue (clone 3) resulting in preferential annealing between these DNA strands to the exclusion of the other clones.

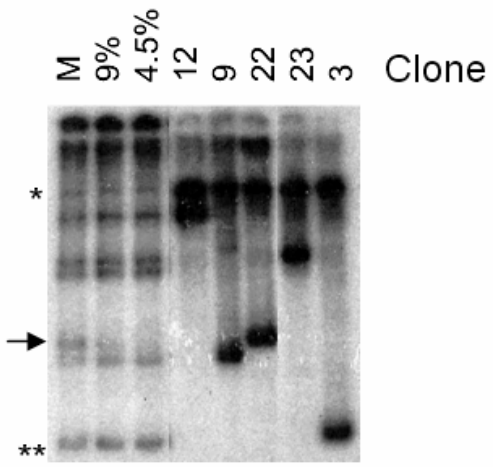


Figure 3.6. HTA demonstrating that detection of individual genotypes can be lost if the variant does not constitute at least 10% of the sample. SIV/DeltaB670 clone 22 was titrated to represent 9% and 4.5 % of the total sample. The arrow represents the clone 22 position. The V1-V2 envelope region was amplified from individual plasmids and tracked with SIV/DeltaB670 clone 3. The asterisk and double asterisk indicate the single strand probe and homoduplex position respectively. M indicated a mix of the six individual clones, clone numbers are given across the top of the gel. SIV/DeltaB670 clone 6 is not represented individually but migrates to the position under clone 22.

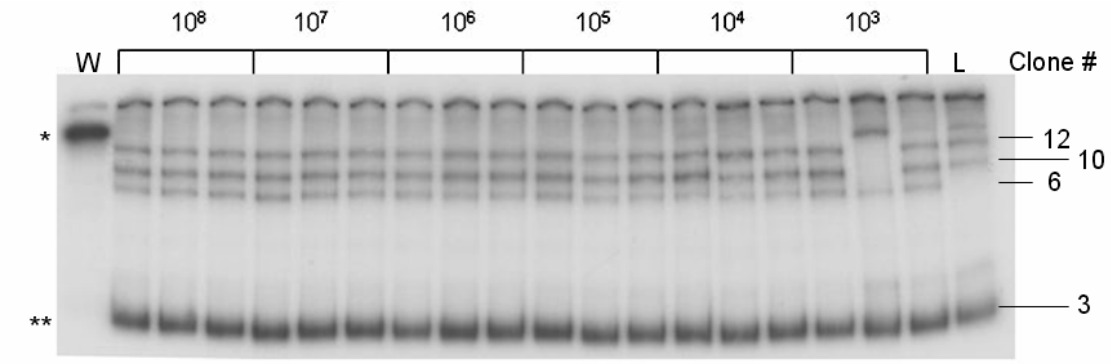


Figure 3.7. HTA representative of variable plasma virus loads. Equal copy numbers of four plasmids containing SIV/DeltaB670 clones (12, 10, 6, and 3) were mixed to total the copy numbers indicated at the top of the figure. The template copy number was calculated based on the optical density of the plasmid preparation. The four clones were serially diluted to 1/4 of the desired copy number, mixed and amplified by PCR. The asterisk and double asterisk represent the single stand and homoduplex positions respectively. W is the probe with water alone, L is a clone ladder with the individual clones identified on the right.

***Discussion***.

The ability to monitor virus evolution within an infected host provides us the opportunity to potentially correlate genotypic changes with pathogenic events. Heretofore this has been an arduous, expensive task requiring the cloning and sequencing of a large numbers of samples. The time and resources involved can limit the analysis and result in an incomplete survey. We have developed the heteroduplex tracking analysis (HTA) for use with our SIV isolate, DeltaB670. This assay has the ability to evaluate a large number of samples in a timely manner.

The amount of sequence diversity within the V1 region from the SIV/DeltaB670 clones allowed us to select the viral genotype to track over infection. The use of clone 3 provided a clear distinction between genotypes in this analysis. Studies have shown sequences differing less than 1.5% (approximately 7 nucleotides in our assay) will form heteroduplexes without reduced mobility (75, 329) and clone 3 contains a large deletion when compared to the other DeltaB670 genotypes. The lack of annealing partners is believed to cause the unpaired DNA to loop out and cause kinks in the double strand that reduce the mobility further than mismatched base pairs (23). We did not test the clone 3 probe against all of the genotypes present within an inoculum therefore other genotypes may have the identical mobility when duplexed with clone 3. This result is possible but we feel it is unlikely to create a problem because we want to track global changes within the virus population rather than individual genotypes.

Our detection limit for rare variants was 10% as has been previously reported (168). We feel this level of detection is adequate for determining the effects of antiretroviral strategies on virus evolution because any genotype representing less than

10% of the population does not have a replication advantage over the other genotypes at the time of sampling. We additionally demonstrated that a plasma virus burden of 1 X $10^3$ was necessary for reliable quasispecies sampling.

The rapid mutation rate of this virus results in the emergence of variants that are able to escape recognition by the host's immune response thereby limiting the effectiveness of the response. The optimization of HTA for use with our primary SIV isolate has enabled us to monitor genotypic changes in the virus population *in vivo*. This ability can provide insight into the influence of antiretroviral drug therapy and vaccine strategies on virus evolution. With this knowledge, newer antiretroviral strategies can be designed to target specific genotypes that demonstrate an increased fitness or pathogenicity.

# Chapter 4. Effects of Monotherapy with (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) on the Evolution of a Primary Simian Immunodeficiency Virus (SIV) Isolate.

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***Abstract***

Determining the impact of antiretroviral therapy on virus evolution could advance the development of improved therapeutics/vaccines against HIV. Toward this goal, we analyzed virus burden, quasispecies complexity, and T cell responses in SIV/DeltaB670-infected rhesus macaques +/- treatment for 7 months with PMPA (2-30 weeks post-infection). Treatment divided the animals into two groups: poor responders (a reduction of $\leq$1 log) and responders ($\geq$ 2 log reduction) in virus burden. Virus evolution in poor responders and untreated controls was characterized by expression of a complex quasipecies that evolved as the disease progressed. This included the universal loss of a viral genotype selected against by *in vitro* passage in monkey cells and selected for by propagation in human cells. In contrast, a good response to PMPA was characterized by infection with a less complex quasipecies that evolved more slowly. Interestingly, in 2 of the best responders, the human-preferred genotype persisted until the study was discontinued (89 weeks p.i.). Neither virus burden nor the magnitude of the T cell response at 2 weeks post infection predicted PMPA responsiveness. However, responders expressed a less complex quasispecies than nonresponders prior to treatment. These data suggest a role for intrinsic host factors in treatment responsiveness, and lend support for therapeutic vaccination as an adjunct to effective therapy.

***Introduction.***

Treatment of HIV infected individuals with one or more drugs that interfere with the viral life cycle has provided a significant therapeutic benefit by reducing the clinical symptoms and prolonging the life of infected individuals (83, 91, 233), These therapies are not perfect, however, because they do not eradicate the virus (52), and treatment fatigue and/or the emergence of drug resistant strains eventually occurs (20, 64) which frequently heralds an increase in virus burden and a return of symptoms. Additonally, not all people respond well to therapy (114, 146). What is needed to offset these shortfalls is the development of strategies that, when given in combination, augment the effects of therapy, and allow continued control once treatment is terminated. A better understanding of the impact of drug treatment on the virus and how host factors influence this process is essential to these efforts. In infected humans, such studies are confounded by the extent of HIV genetic variation and sorting out the multiple selective pressures that drive viral evolution. The exhaustive genetic analysis of HIV strains isolated across the globe and longitudinally over time in numerous infected individuals has provided some insight into this process. Foremost in these events is the evolution associated with the ongoing adaptation of the virus to replication in the host ("fitness")(40). Second, is the influence of immune selection of nascent genotypes capable of evading either B (8, 205) or T cell immunity (9, 36, 113, 242). Together, these forces play a collective role in driving divergence and diversity in the virus population over time (181, 262). More recently, the selection of drug resistant strains as a consequence of long term antiretroviral therapy has also impacted HIV evolution. Drug resistant viruses, however, are often poorly competitive with their drug sensitive counterparts and are lost during the

viral rebound that follows treatment interruption (84, 85, 148). Interestingly, the drug sensitive genotypes expressed during rebound are often more closely related to viruses replicating during early infection (ancestral strains) than the more recently expressed drug resistant genotypes. This finding has promoted the concept of organ and/or cellular reservoirs of latent virus that provide a continual source of virus during chronic infection (53, 325).

Similar evolutionary pressures exist in the SIV:macaque model for AIDS as well. Evasion of T cell immunity via selection of epitope escape mutants is well described in the infected macaque (49, 93, 229) as well as the emergence of drug resistant strains during antiretroviral therapy (298). Adaptation to growth in different species most certainly occurs as well. Many of the virus stocks used today that were originally isolated from the Sooty Mangabey monkey, have been extensively passaged in the rhesus macaque (67, 79, 214, 269). SIV is also frequently propagated in human cell lines for use in *in vitro* assays and the preparation of virus stocks for *in vivo* studies. This recurrent switching of primate hosts provides an ideal environment of genetic instability for SIV that promotes a continuous state of viral evolution independent of host immune selection. A rapid method for determining the quasispecies complexity within a virus population is through the use of a heteroduplex tracking analysis (HTA). This assay is based on the differential migration of duplexed DNA through a highly resolving gel matrix. In this report, we used this knowledge and a virus stock whose passage history and genetics are known (16) to provide insight into lentiviral virus:host:drug interactions by analyzing the virological response to monotherapy with the nucleotide analog (R)-9-(2-phosphonomethoxypropyl) adenine (PMPA).

*Materials and Methods*

**V1-V2 *env* PCR amplification.** The V1-V2 region of the envelope gene was amplified from plasma derived cDNA to monitor viral evoluation. Nested PCR reactions were initiated immediately following completion of the duplicate reverse transcription reactions. A 30 μl PCR master mix containing 1 X PCR buffer II, 2 mM MgCl2, 2.5 U Amplitaq Gold (Perkin Elmer, Wellesley, MA), 200 mM dNTPs, and 20 pmol each of the first round primers was added directly to the reverse transcription reactions. The first round primers were 5'-AGG-AAT-GCG-ACA-ATT-CCC-CT-3' (SIVmac239 nucleotides 6709 to 6728) and 5'-TCC-ATC-ATC-CTT-GTG-CAT-GAA-3' (nucleotides 7406 to 7385). The amplifications were carried out in an Applied Biosystems Gene Amp PCR System 9700 (Foster City, CA) by heating at 95 °C for 10 min, followed by 27 cycles of 95 °C for 15 sec, 55 °C for 15 sec and 72 °C for 30 sec. The 100 μl second round reaction was carried out with 2 μl of the first round reaction, 1 X HotMaster Taq Buffer with $Mg^{2+}$, 200 mM dNTPs 20 pmol each second round primer and 3 U HotMaster Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The amplification was initiated by heating at 95 °C for 2 min and completed using the first round cycling parameters. The second round primers were 5'-CAG-TCA-CAG-AAC-AGG-CAA-TAG-A-3' (SIVmac239 nucleotides 6845 to 6868) and 5'-TAA-GCA-AAG-CATAAC-CT-GCG-GT-3' (nucleotides 7305 to 7327). PCR products from duplicate positive reactions were pooled and the resulting 480-bp fragments were used in either the tracking analysis or cloned for sequencing.

**Rhesus macaques.** Macaques were maintained in accordance with the NIH Guide to the Care and Use of Laboratory Animals under the approval of the University of

Pittsburgh Institutional Animal Care and Use review committee. The University of Pittsburgh is accredited by the American Association for the Accreditation of Laboratory Animal Care International. These animals were part of a larger cohort of animals used in a therapeutic vaccine trial (102). The Mamu class I haplotype was determined by PCR-SSP and direct sequencing as previously described (153).

**Viral challenge**. Macaques were fasted for 12 hours, sedated with ketamine and challenged intravenously with 1 ml of RPMI containing 10-100 $TCID_{50}$ of cryopreserved SIV/DeltaB670. Clinical status was monitored by complete blood cell counts and physical examination consisting of palpation of peripheral lymph nodes and spleen and monitoring for signs of opportunistic infections. CD4+ T cell counts were measured by flow cytometry after staining of whole blood as described (190).

**Anti-retroviral therapy.** Rhesus macaques received daily subcutaneous injections of the anti-retroviral (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) (Gilead Biosciences, Foster City, CA) at a dosage of 20 mg/kg starting 2 weeks after infection. Treatment was continued without interruption for 28 weeks and then discontinued to monitor the effects of therapy on viral rebound and progression to disease.

**In vitro propagation.** The original stock of SIV/DeltaB670 consisted of the culture supernatant derived from co-cultivation of a lymph node homogenate from monkey B670 with rhesus monkey PHA blasts (214) passage, the cells were washed and resuspended in fresh medium on day 11 post infection, and the supernatant harvested three days later for genetic analysis.

Virus obtained from the second passage was used to inoculate the monkeys in the study and for passage in CEMX174 cells. One million CEMX174 cells were inoculated with 100 TCID50 of the second passage, the cells subcultured every 3 days. The culture supernatant used for genetic analysis was harvested 21 days post infection.

**Reverse transcription.** Synthesis of cDNA was performed in duplicate reactions containing 5 MgCl, 1 X PCR buffer II, 0.75 mM dGTP, 0.75 mM dATP, 0.75 mM dCTP, 0.75 mM dTTP, 1 U RNase inhibitor, 1.2 U MULV reverse transcriptase (RT), 2.5 μM random hexamers using10% of the total viral RNA obtained from high speed plasma pellets. Samples were incubated at room temperature for 10 minutes, followed by 42 °C for 12 min, and the reaction terminated by heating at 99 °C for 5 min. Samples were cooled to 4 °C prior to PCR amplification.

**Plasma viral loads.** Virion-associated RNA in plasma was quantified by real-time RT-PCR using 10% of total RNA in a Prism 7700 (Applied Biosystems, Inc., Foster City, CA) using primers specific for the viral long terminal repeat as described (101). This assay is linear over an 8-log range of template copy number and has a sensitivity threshold of 10 copies/reaction. Control amplifications of samples omitting reverse transcriptase yielded negative results. RNA copy numbers from the unknown plasma samples were calculated from a similarly amplified external standard and expressed as RNA copies/ml plasma.

***Pol* PCR amplification and sequencing.** Nucleotides 2985 to 3140 of the polymerase gene were amplified from viral cDNA in duplicate nested PCR reactions as previously described (184), with the exception that HotMaster Taq DNA polymerase was

used for amplification (Eppendorf, Hamburg, Germany) using 10% of total RNA. PCR resulted in a 156-bp fragment encoding RT amino acids 52 to 90.

**Heteroduplex tracking assay.** Nucleotide changes in the V1-V2 region of *env* were monitored using a heteroduplex tracking assay (HTA). HTA was performed using the following modifications of the procedure originally described by Delwart and Gordon (76). The PCR reactions were pooled, gel purified (QIAEX II gel extraction kit, QIAgen, Valencia, CA) and eluted in 50 μL water. Amplification and gel purification was repeated using 1 ng of the gel purified product as starting material.

The radiolabeled probe was generated by asymmetric amplification of 10 ng of the gel purified product in a 100 μL reaction using only the second round forward primer. The cycling conditions were: 1 X PCR buffer II, 50 mM $MgCl_2$ 0.75 mM dCTP, 0.75 mM dTTP, 0.75 mM dGTP, 1:6 diluted dATP, 10 μCi $^{32P}$ dATP, 2.5 U Amplitaq gold. The resulting radiolabeled single strand probe was subjected to electrophoresis at 60 mV for approximately 3 hours, visualized by autoradiography, excised, gel purified, and stored at –20 °C until use.

Fifteen μl of pooled V1-V2 PCR products were individually mixed with 5 μl of a master mix containing 1 μl of radiolabeled single strand probe, 2 μl HTA buffer (1 M NaCl, 100 mM Tris, pH 7.4, 20 mM EDTA) and 2 μl water. The mixture was heated to 93 °C for 3 min and immediately placed on ice. The duplexed DNA was subjected to electrophoresis in 5% MDE (Cambrex Bio Science, Rockland, ME) in 1X TBE buffer (1 M NaCl2, 100 mM Tris pH 7.5, 20 mM EDTA) at 300 V for 3.5h. The gel was dried and the bands were visualized by autoradiography.

**Sequence analysis.** Duplicate PCR reactions were pooled and the resulting fragments cloned into the pcDNA3.1/V5-HIS-TOPO TA cloning vector (Invitrogen, Carlsbad, CA) for sequencing. Five to 20 clones were randomly selected and sequenced in an ABI 370 automated DNA sequencer (Applied Biosystems). Alignment of the sequences was performed using CLUSTAL X (284).

**Estimation of potential N-linked and O-linked glycosylation sites.** V1-V2 sequences were analyzed for potential N-linked glycosylation sites by visual inspection for the sequence motif NXT/S. Potential O-linked sites were determined using the program NetOGlyc (available at http://www.cbs.dtu.dk/services/NetOGlyc-2.0/ (121). All potential O-linked sites with a G-score above 0.5 were included in the analysis. No sites (S or T residues) were found to have a prediction above 0.758. The potential glycosylation sites in the inoculum were determined using the published sequences for SIV/DeltaB670 (16)

**Proliferation assay.** Virus-specific T cell proliferative responses following stimulation with whole SIV gag (Trinity Biosciences, Frederick, MD) and tat (kind gift, Dr. David Watkins, University of Wisconsin) recombinant proteins were measured as previously described (102).

**IFN-γ ELISPOT Assay**. SIV-specific CD8+ T cells secreting IFN-γ were enumerated by ELISPOT following stimulation with a synthetic epitope peptide (QCB, Hopkinton, MA) or Gag and Tat peptides pools consisting of 15-mers overalapping by 11 amino acids (Chiron, Emeryville, CA) as previously described (102).

**Statistical analysis.** Data from the untreated, poor responders and responders

were compared by the Mann-Whitney U test. All *P* values reported are two-sided. In all cases, $P < 0.05$ was considered significant.

***Results***

*Study Design.* Virus burden, quasispecies evolution, and SIV-specific T cell responses were evaluated in twelve SIV/DeltaB670 infected rhesus macaques temporally treated with PMPA to identify factors associated with the response to treatment and rebound after treatment interruption. SIV/DeltaB670 is a primary virulent isolate that causes AIDS in macaques with a mean time to death of 11 months (261). Treatment was initiated at 14 days post inoculation (pi) in 8 monkeys and continued for a period of 28 weeks at which time it was discontinued. The remaining 4 animals served as untreated infected controls.

Treatment was initiated during acute infection because SIV infection in macaques is more aggressive and causes death more rapidly than HIV in humans (197, 276, 309). Thus, this schedule should mimic the effects of initiating HAART during later stages of infection in HIV infected patients. A single drug was employed for this study because a cocktail of drugs administered during acute infection with SIVmac251 prevented viral rebound in some of the animals after treatment was withdrawn (125) and it was imperative for this study that viral rebound occur after discontinuation of the drug.

*Clinical disease course.* The disease course in these animals is summarized in Table 1. As expected, all four untreated infection controls died by 474 days pi with an AIDS-defining illness. Twenty-eight weeks of PMPA therapy significantly prolonged survival in the treated group ($P = 0.048$, Mann-Whitney U, two-tailed) with only two of these animals dying of AIDS within this timeframe. The six surviving monkeys were sacrificed for tissue collection 589-664 days pi.

TABLE 1. Study Outcome of SIV/DeltaB670 Infected Untreated and PMPA Treated[a] Animals

| Group | Animal Number | Survival (Days) | Mamu Haplotype | Cause of Death |
|---|---|---|---|---|
| Untreated control | M11599 | 101 | A*08,B*03 | CMV pneumonia |
| | M10699 | 474 | A*08 | SIV giant cells in lung |
| | M10399 | 360 | A*02 | Meningitis |
| | M4599 | 365 | B*01, B*03 | PCP pneumonia, cryptosporidium |
| PMPA, poor responder | M10899 | 240 | B*01 | CMV, Lymphosarcoma |
| | M9699 | 589[b] | A*01, A*02 | Euthanasia |
| | M11399 | 394 | Unknown[c] | Lymphosarcoma, meningitis |
| | M12499 | 664[b] | A*01, A*02 | Euthanasia |
| PMPA, responder | M10999 | 636[b] | Unknown[c] | Euthanasia |
| | M9099 | 662[b] | A*01 | Euthanasia |
| | M9799 | 660[b] | A*08,B*01, B*03 | Euthanasia |
| | M13799 | 664[b] | A*01,B*01, B*03 | Euthanasia |

[a] Daily PMPA treatment (20 mg/kg) initiated 14 days pi, continued six months then withdrawn
[b] Scheduled sacrifice
[c] Haplotype was not Mamu; A*01, A*02, A*08, A*11, B*01, B*03, B*04, B*17

Because recent reports had shown that lower viral loads and disease resistance were associated with the Mamu-A*01 class I allele in monkeys infected with SIVmac239 (211, 232). Among these animals, there was no apparent influence of this allele on either survival or virus burden and drug responsiveness (see below). This result is consistent with the observations of a larger cohort of Mamu-A*01 positive animals infected with SIV/DeltaB670 (102) and suggests that the Mamu-A*01 effect on disease may be dependent on the viral isolate employed.

*Virus burden.* The virus burden in untreated infected controls was typical of SIV/DeltaB670 infected rhesus macaques, with peak virus loads of $10^6$-$10^8$ RNA copies/ml by 14 days pi followed by a decline to a setpoint by 4-8 weeks pi of $10^5$ – $10^7$ RNA copies/ml and a plasma virus burden above $10^5$ RNA copies/ml during chronic

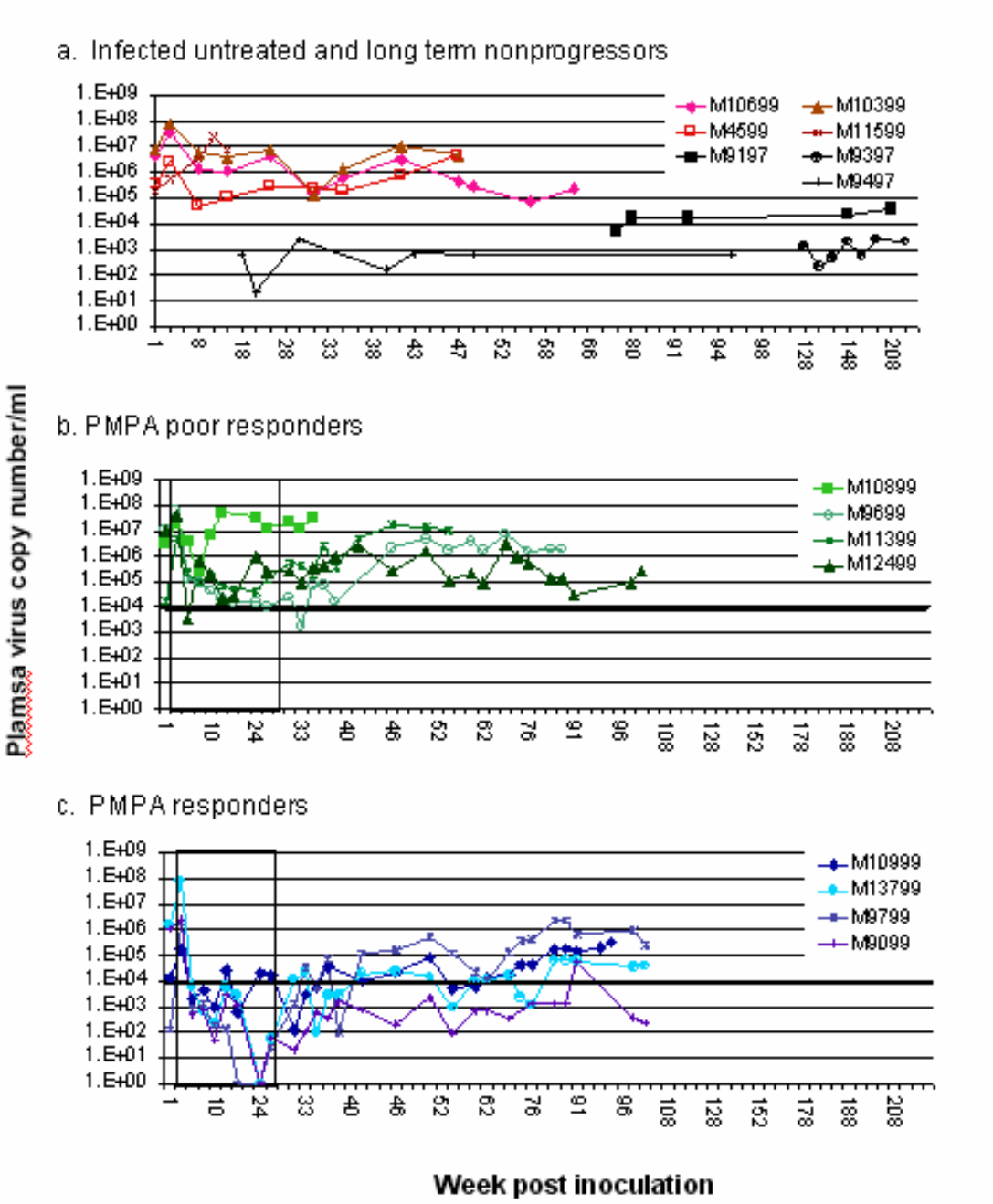



Figure 4.1. Plasma virus load for infected untreated (a) and PMPA-treated animals (b, poor responders and c, responders) over time postinoculation. Untreated animals (a) are represented as progressors (grey lines) or long-term nonprogressors [LTNPs (black lines)]. The vertical line represents the average plasma virus burden from LTNPs ($10^4$ copies/ml). Animals were designated as treatment poor responders (b) or responders (c) based on maintenance of an average virus load during treatment to or below that of an LTNP. Plasma was serially sampled and virus copy number/ml plasma was calculated by real-time RT-PCR. Cutoff for the assay was 10 copies/ml. Boxed area represents treatment period (weeks 2–30 postinfection).

infection (Figure 4.1a). These levels are in contrast to that observed in long-term nonprogressors (LTNP) infected with SIV/Deltab670 where the virus burden during chronic infection remains at or below $10^4$ RNA copies/ml (Figure 1a). Because LTNP typically live for long periods in a clinically asymptomatic state, this value was used as an efficacious endpoint for therapy.

The virologic response to PMPA varied significantly among the animals, with half of the animals responding poorly to treatment (poor responders: Figure 4.1b) and half responding well (responders: Figure 4.1c). In poor responders the virus burden declined to one log or less than that observed in untreated controls, with the virus load in one animal rebounding during therapy. These values were not significantly different from that in the untreated controls ($P = 0.39$, Mann-Whitney U, two-tailed). These results are in contrast to those obtained in the 4 responder monkeys where the virus loads were reduced to or below the levels typically observed in LTNP monkeys (responders; Figure 4.1c). PMPA therapy in three of these animals further reduced the virus burden below our detection limit, an outcome we have not seen in untreated SIV/DeltaB670-infected macaques (unpublished). These values were significantly lower than those observed in untreated controls ($P = 0.029$, Mann-Whitney U, two-tailed). Although a similar dichotomy in response to therapy has been observed in HIV-infected humans (114, 146), the differences observed in our study were surprising given that the virus inoculum and PMPA dose were the same, and the interval between infection and initiation of therapy remained constant.

Discontinuation of PMPA treatment at 30 weeks p.i. eventually resulted in viral rebound in all eight animals. The post-therapy virus burden, however, differed between

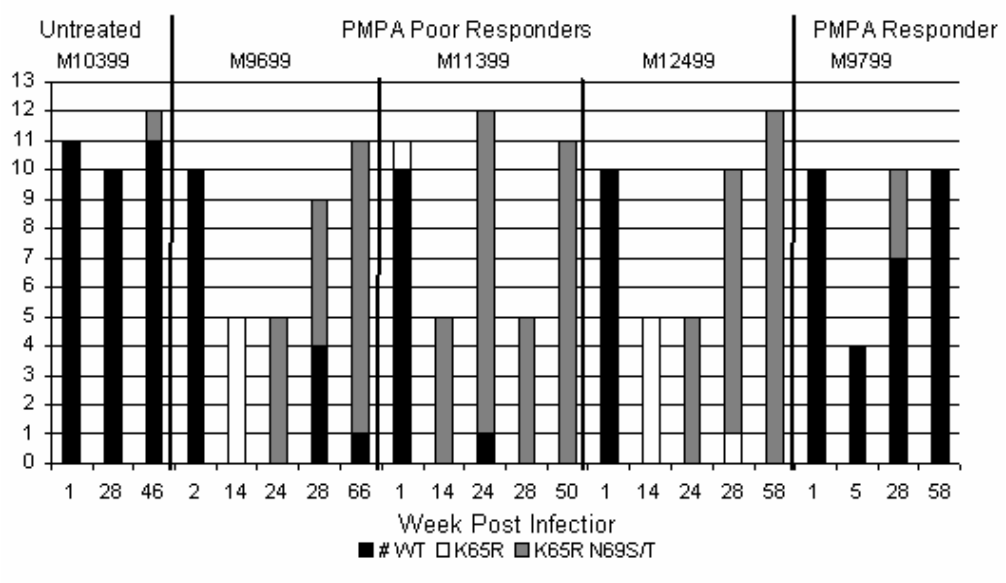



Figure 4.2. Number of clones expressing wild type or drug resistant reverse transcriptase (RT) from plasma virus. A portion of the reverse transcriptase gene was amplified from plasma viral cDNA at the indicated time postinoculation and sequenced. Black bars represent wild type RT, grey bars represent RT containing the K65R mutation, and white bars represent RT with both K65R and the compensatory N69S/T mutation.

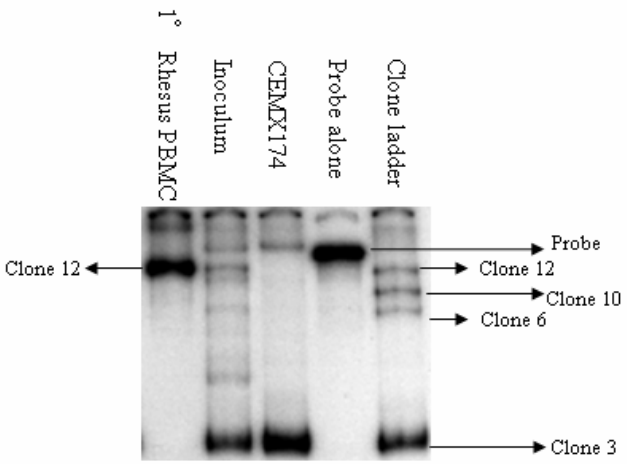



Figure 4.3. HTA of V1-V2 region from: lane 1, viral cDNA from SIV/DeltaB670 passaged in primary rhesus PBMCs in vitro; lane 2, viral cDNA isolated and amplified from an SIV/DeltaB670 inoculum; lane 3, viral cDNA from in vitro passage in human T cell line (CEMx174); lane 4, probe alone; lane 5, V1-V2 amplified from plasmids containing the indicated clones and combined.

responders and poor responders, with the values at weeks 32 and 34 achieving statistical significance (P = 0.03, Mann-Whitney U, two-tailed). When compared to the values observed in untreated controls, the virus burden in the poor responders was not significantly different even though virus rebounded in three of these animals soon after treatment interruption (Figure 4.1b). These results are in contrast to that observed in the PMPA responders which remained below $10^5$ in three animals for over a year after treatment was withdrawn. Virus eventually rebounded in one responder animal (M9799) 10 weeks after treatment interruption to a level achieved in untreated animals.

*Evolution of PMPA resistance.* In previous studies with PMPA treated macaques infected with SIVmac251, a lysine to arginine substitution at amino acid 65 was identified in the pol gene that conferred a 5-fold resistance to PMPA *in vitro* (298), but these were believed to be involved in increasing viral fitness because these mutations were not associated with increased drug resistance. The nucleotide sequence of this region was therefore serially monitored in this study in one untreated control (M10399), three poor responders (M11399, M9699, and M12499), and the PMPA responder animal (M9799) that rebounded above $10^5$ copies/ml to determine the relationship of drug responsiveness and PMPA resistance (Figure 4.2). With the exception of one clone, only the wildtype PMPA sensitive genotype was detected during acute infection in any of the animals. During therapy, however, resistance mutations readily dominated the infection of all three poor responders. These changes included not only the K65R primary resistance mutation, but also a N69S/T mutation (298). Interestingly, the K65 and N69S/T mutations became fixed in the virus population in all three of these animals, and remained as the dominant genotype 16 months after discontinuation of the drug. In

contrast, these mutations were only transiently detected in the responder animal M9799. By 58 weeks pi, only the wild type sequence could be detected even though the virus burden had rebounded to the level achieved in untreated animals.

*In vitro characterization of replication fitness of SIV/DeltaB670 genotypes in human and macaque cells.* Heteroduplex tracking analysis (HTA) was employed for analysis of the viral quasispecies due to its proven utility in epidemiologic studies to distinguish between multiple HIV (75, 78) and SIV (115, 251) genotypes, and the ease with which a large number of samples can be analyzed. The V1-V2 region of *env* served as the target for these assays because it is the most hypervariable region of the genome (231) and thus should provide a sensitive indicator of viral evolution. The SIV/DeltaB670 stock used for these experiments is comprised of over 50 replication competent genotypes as defined by sequence heterogeneity in V1-V2 (16)( Figure 4.3, lane 2), with 2 genotypes, B670 clones 3 and 12, dominating [62 and 10%, respectively; (252)]. A single passage in CEMX174 cells, a T cell line of human origin that is highly permissive for replication of SIV, resulted in the selection of only B670 clone 3 (Figure 4.3b, lane 3). In contrast, three successive passages in rhesus primary PBMC resulted in the selective outgrowth of only the B670 clone 12 genotype (Figure 4.3b, lane 1). These results were confirmed by sequence analysis of over 30 cloned PCR fragments derived from a pool of multiple PCR reactions of each sample (data not shown). As predicted from our *in vitro* observations, direct serial monkey-to-monkey passage of this stock in rhesus macaques clonally selected for B670 clone 12 (129). Infection of macaques with passaged virus was associated with higher virus loads, faster disease progression, and shortened survival. Together, these data demonstrate that propagation of SIV/DeltaB670

in human and macaque cells is associated with selective, rapid outgrowth of genotypes specific for each host. Loss of the B670 clone 3 genotype by passage in macaque cells or, conversely, selection of this genotype by passage in human cells, provides a dramatic display of the selective forces within each species that drive viral divergence independent of immune selection.

*Viral genetic evolution in vivo.* The selection of specific genotypes made apparent by our *in vitro* studies prompted us to evaluate the impact of PMPA therapy on this process *in vivo*. Using the B670 clone 3 as the probe, HTA was performed on cDNA from longitudinal plasma samples from all the animals in the study. In untreated macaques, intravenous inoculation with the primary stock resulted in variable patterns of expression of multiple genotypes clearly discernable at 1 week pi, with either one or both of the dominant variants (B670 clones 3 and 12), among others, detected (Figure 4.4). As expected from our *in vitro* experiments, by 30 weeks p.i, the B670 clone 3 genotype was lost in all of the animals. By 10 weeks pi, changes in the quasispecies were additionally visible in three of the animals, with the appearance of new genotypes observed in some (M10399 and M10699, Figure 4.4 b and c) and shifts in the dominant genotype observed in others (M11599 and M4599, Figure 4.4a and c). As time progressed, the quasispecies became more complex, with multiple new genotypes expressed at the terminal stages of the disease. The one exception to this pattern was M11599 (Figure 4.4a) where the loss of B670 clone 3 was the only major change observed. This observation was not unexpected given that this animal exhibited a "crash and burn" phenotype characterized by a high persistent virus burden, rapid disease progression, and shortened survival (324). Animals exhibiting this phenotype fail to mount an immune response to SIV and are thus

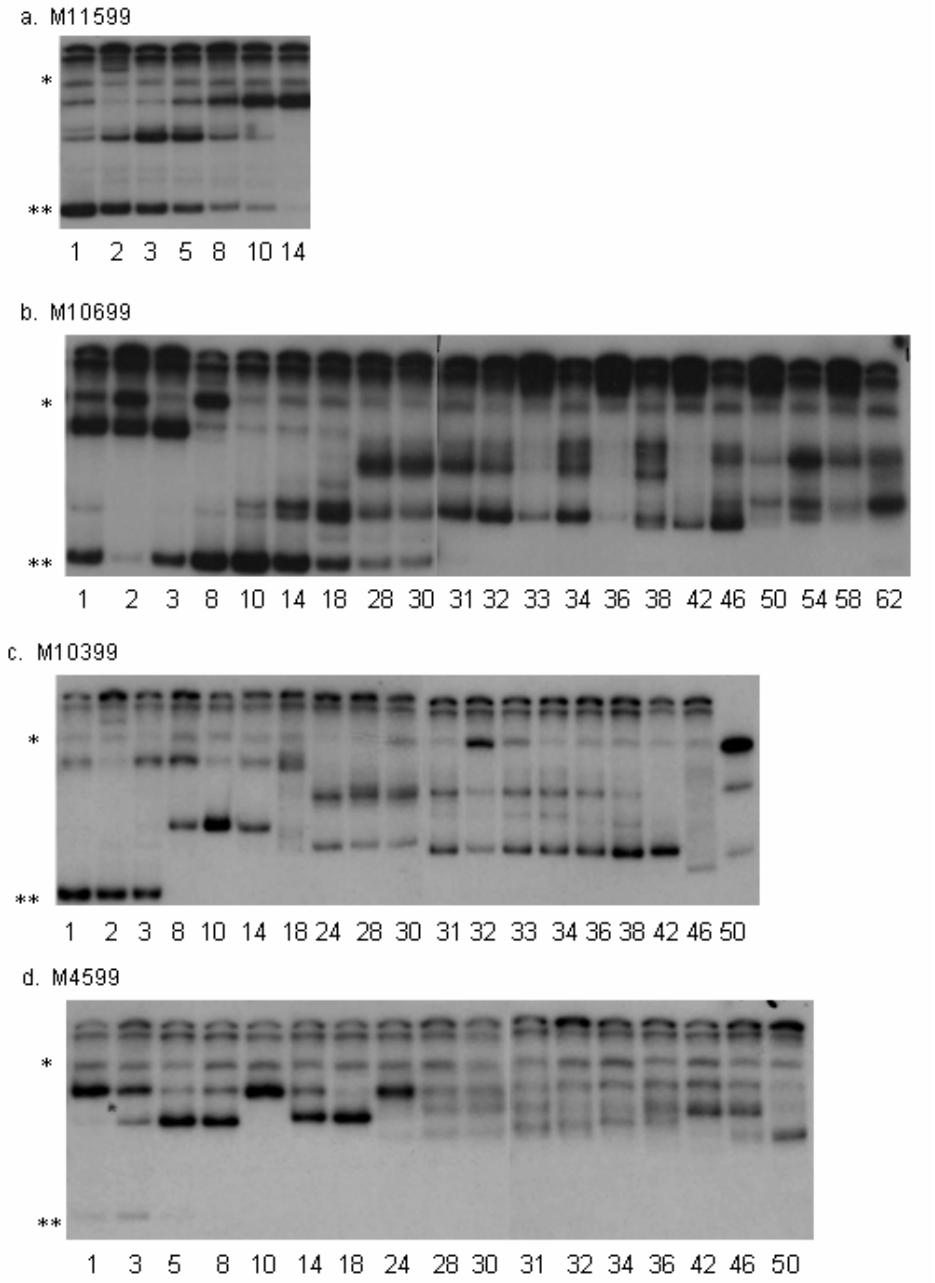



Figure 4.4. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, untreated macaques. Numbers along the bottom indicate week postinoculation. The last week is the necropsy sample. The V1–V2 envelope region was amplified by nested PCR. The resulting amplicons were hybridized to a SIV/DeltaB670 clone 3 V1-V2 radiolabeled probe as described to track changes in this region. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. The top two bands in each lane are PCR remnants and are not included in the enumeration of variants. (a) M11599; (b) M10699; (c) M10399; (d) M4599.

presumably unable to drive evolution by immune selection. Phylogenetic analysis of V1 sequences obtained over time in representative animals confirmed that the emergence of new genotypes revealed by HTA was indeed in part due to evolution of the input virus (data not shown).

Macaques that responded poorly to PMPA (Figure 4.5) showed a pattern similar to that observed in untreated animals. Multiple genotypes were detected during acute infection, and an increase in the complexity of the quasispecies occurred as the disease progressed. This was particularly evident after the discontinuation of PMPA. Loss of the B670 clone 3 genotype was also apparent. It is interesting to note that in two of these animals a major shift in the dominant genotype present in plasma was observed 24 weeks pi (monkeys 9699 and 11399, panels b and c, respectively). Like that seen in HIV infected humans, these data are consistent with the presence of a cellular or organ reservoir that served as a source for this genotype. However, the changes observed did not appear to be associated with either the emergence of PMPA resistance or the discontinuation of therapy because resistant genotypes had emerged before this time (Figure 4.2) and treatment was not terminated until 30 weeks pi.

Monkeys that responded to PMPA exhibited notable differences when compared to poor responders or untreated controls (Figure 4.6). First, in three of these animals (M13799, M9799, and M9099; Figure 4.6b, c and d, respectively) the quasispecies appeared to be less complex. Second, as would be expected by the significant reduction in virus burden associated with effective antiretroviral therapy, virus evolution appeared to progress more slowly as evidenced by continued expression of a relatively unchanged quasispecies. The most striking example of a slowing in virus evolution was the

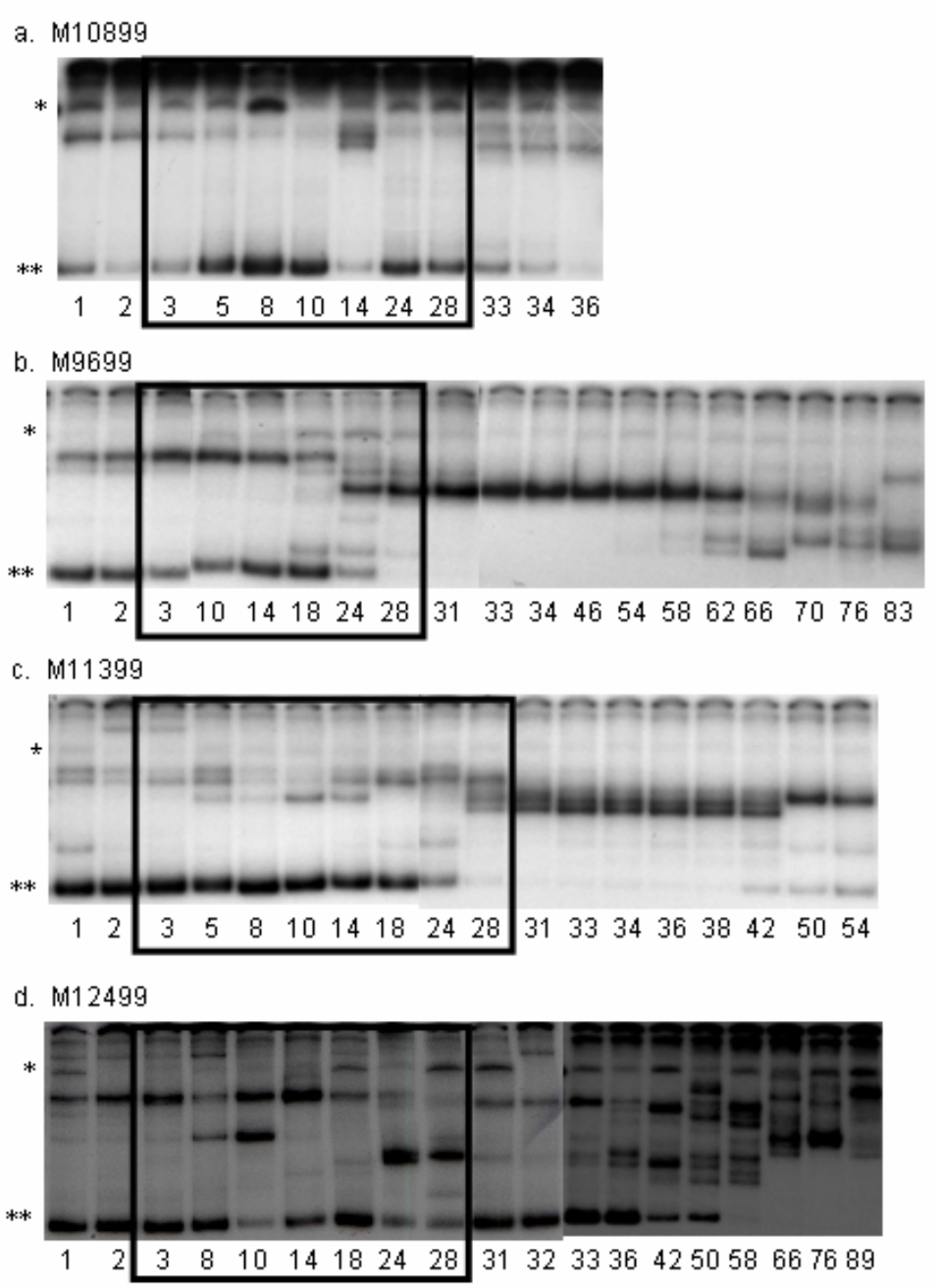



Figure 4.5. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated poor drug responder macaques. Numbers along the bottom indicate week postinoculation. Daily PMPA treatment was initiated 2 weeks pi and continued for 28 weeks. Boxed area represents samples collected during therapy. The last week is the necropsy sample. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. The top two bands in each lane are PCR remnants and are not included in the enumeration of variants. (a) M10899; (b) M9699; (c) M11399; (d) M12499.

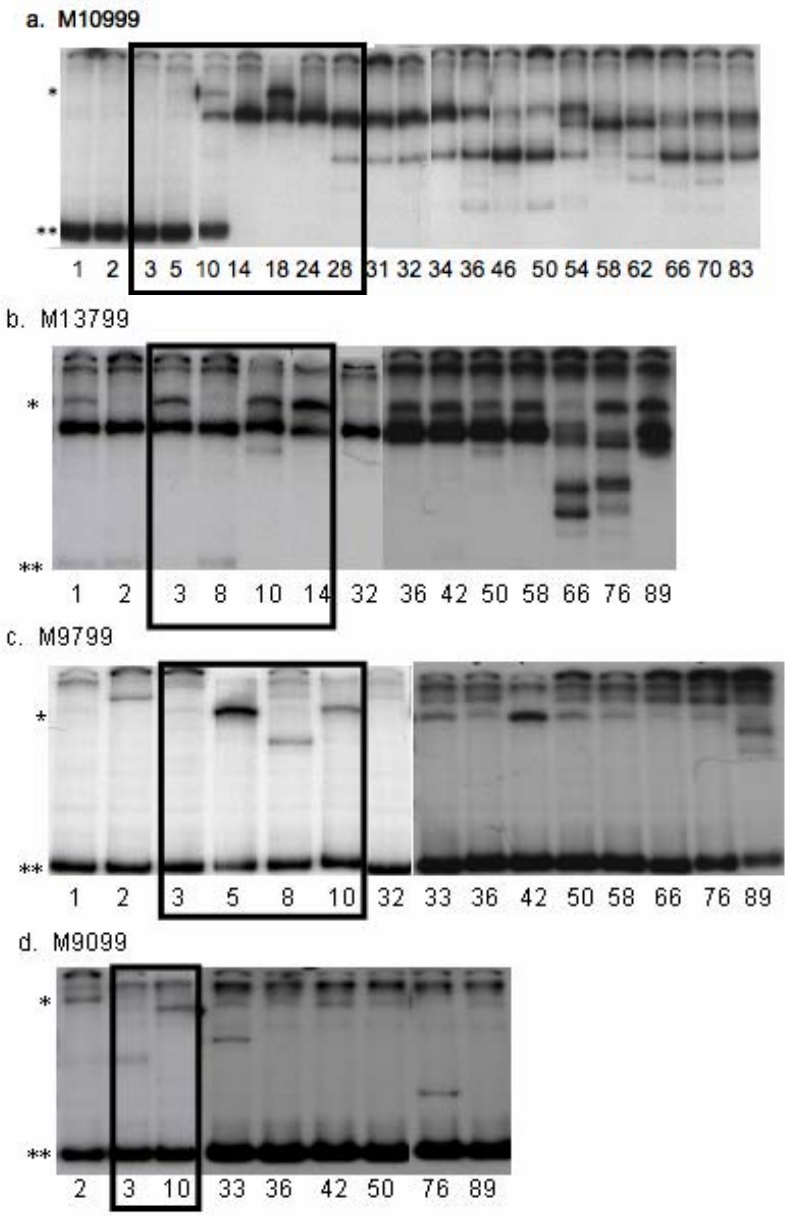



Figure 4.6. HTA of plasma virus cDNA derived V1-V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated responder macaques. Boxed area represents samples collected during therapy. Numbers along the bottom indicate week post inoculation. The last week is the necropsy sample for panels b, c, and d. The necropsy sample for panel a is not shown, however, was indistinguishable from the week 83 sample. The asterisk and double asterisk indicate single strand probe and homoduplex positions, respectively. The top two bands in each lane are PCR remnants and were not included in the enumeration of variants. (a) M10999; (b) M113799; (c) M9799; (d) M9099.

persistence of the B670 clone 3 genotype in two of the animals (monkeys 9799 and 9099; Figure 6 panels c and d, respectively). The limited template copy number of some of the samples analyzed does not fully explain this result because one monkey clonally infected with the B670 clone 3 genotype (M9799) rebounded to $10^6$ copies/ml without a visible change in the HTA. These data demonstrate that not only did effective therapy apparently slow immune escape, it also affected viral adaptation by preventing the loss of a genotype that was poorly fit for replication in macaques. These findings are of particular interest because they suggest that virus can be more easily contained by adjunctive immunotherapy in individuals who respond well to antiretroviral therapy.

*N and O-linked V1-V2 glycosylation sites during infection/therapy.* The acquisition of potential N-linked glycosylation sites in the V1-V2 regions of both HIV-1 and SIV during the course of disease progression has been implicated in the evasion of neutralizing antibody (48, 155, 310), two poor responders (M11399 and M12499) and the PMPA responder that rebounded after therapy (M9799) to determine the impact of infection and therapy on glycosylation of the SIV/DeltaB670 *env* (Figure 4.7). Two potential N-linked glycosylation sites were identified in the V2 region of SIV/DeltaB670; both of these sites were completely conserved among all animals and timepoints analyzed (data not shown). Three conserved N-linked glycosylation sites and a variable fourth site at position 14 were also identified in V1 (Figure 4.7). A comparison of samples from early vs. late time points in untreated and poor responders failed to reveal a consistent pattern of change affecting glycosylation at this position. As expected, fewer changes in position 14 were observed in the responder monkey M9799. However, unlike that seen in the other animals, genotypes at some of the last time points analyzed had lost the last

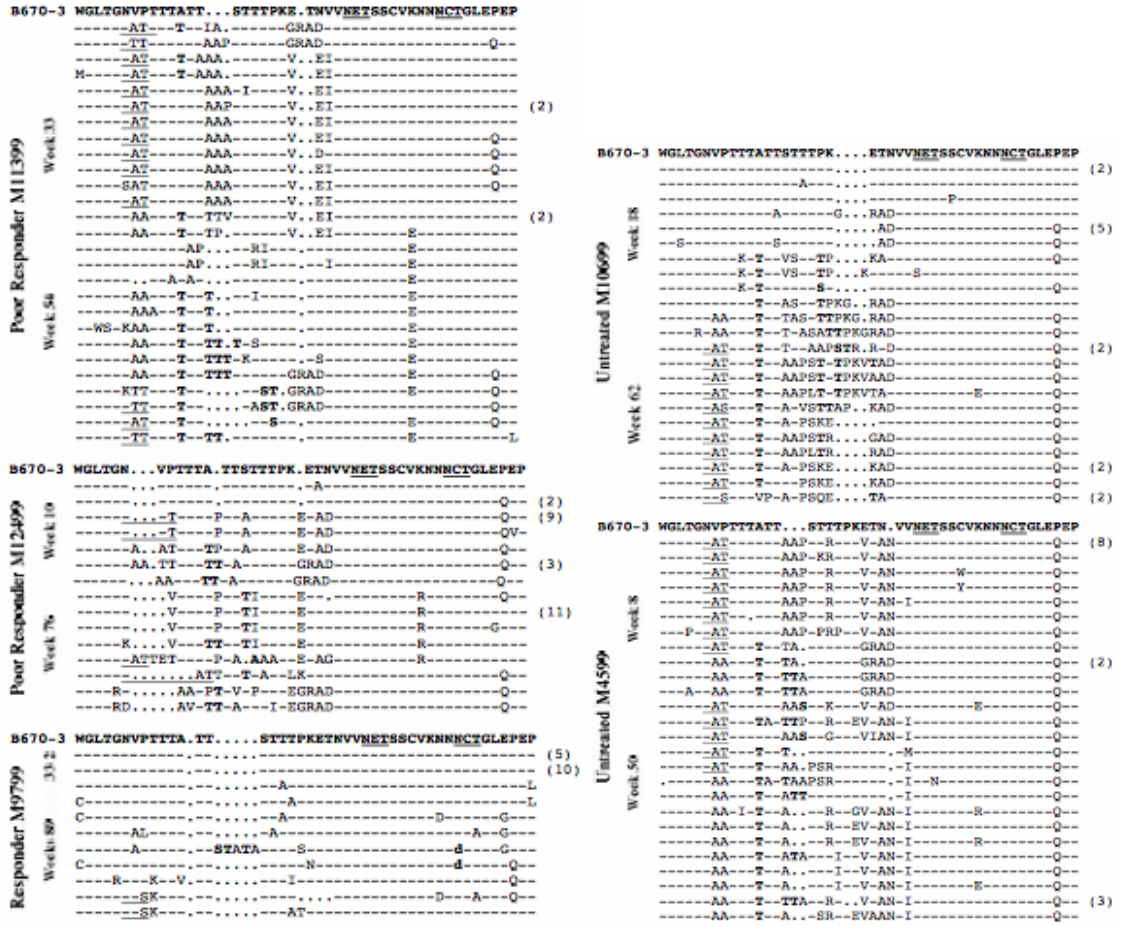



Figure 4.7. Amino acid sequence alignment of the envelope V1 region from untreated and PMPA-treated animals. The V1 region was amplified from viral cDNA at indicated times postinoculation, cloned, sequenced, and aligned with the SIV/DeltaB670 clone 3 consensus (shown in bold). The animal number is preceded by an M, week postexposure is indicated. Dashes indicate identity to clone 3 while dots indicate spaces inserted to aid in alignment. The number of identical clones is given at the end of the sequence. Potential N-linked glycosylation sites are underlined, and acquired potential O-linked glycosylation sites are in bold. The loss of potential Nlinked glycosylation sites is indicated in lower case bold lettering (M9799).

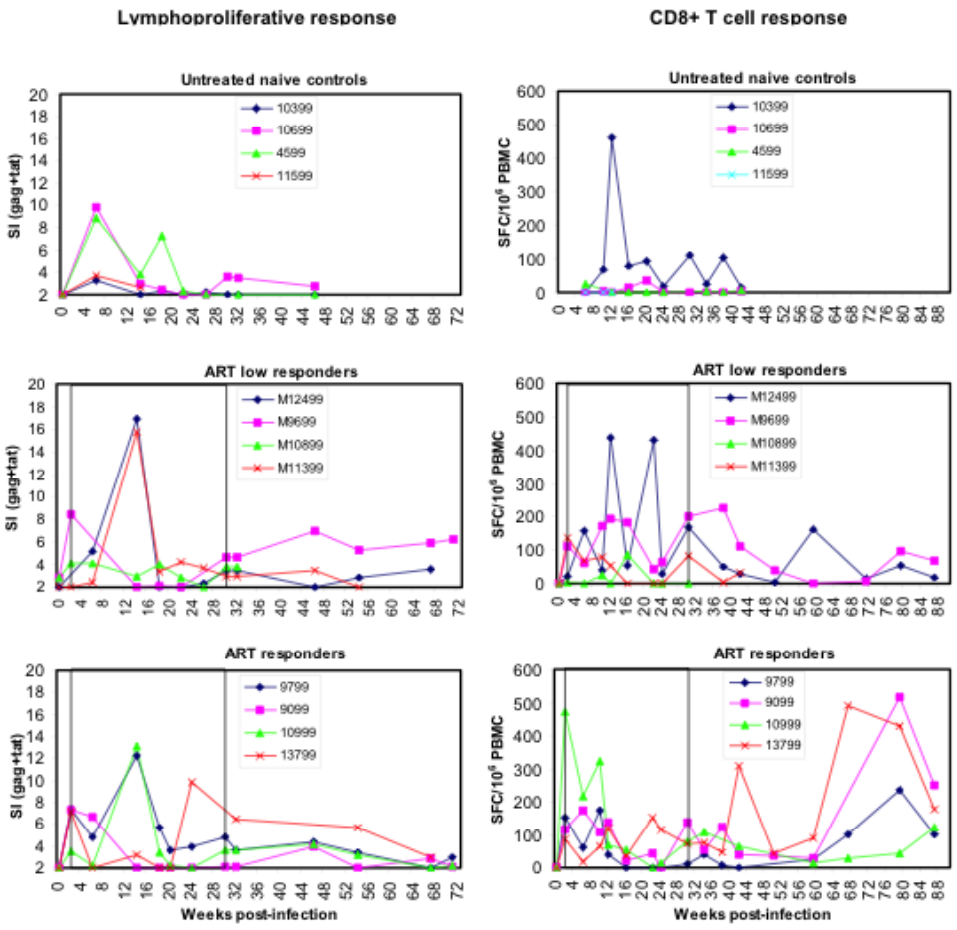



Figure 4.8. Postinfection SIV-specific lymphoproliferative (LPR) (a) and CD8+ T cell responses (b) in untreated naive macaques (top panels), PMPA poor responders (middle panels), and PMPA responders (bottom panels). Shown are individual responses in each animal. SIV-specific LPR was measured following stimulation of freshly isolated PBMC with whole Gag and Tat proteins. SIV-specific CD8+ T cell responses were measured by ELISPOT following stimulation of CD4+ T celldepleted PBMC with overlapping peptide pools encompassing SIV Gag and Tat and 8 representative CTL epitopes conserved in the challenge strain and frequently recognized in Mamu-A*01 positive SIV-infected macaques (10).

potential glycosylation site in V1. Although the significance of this observation is unknown at present, it is intriguing to speculate that this change may be critical to the increase in virus burden observed in this animal, either by providing antibody escape or enhancing fitness. It is notable that, although a direct relationship between function and O-linked glycosylation has not been shown, the hypervariability found in the V1 region identified during infection and disease progression also had a major impact on O-linked glycosylation residues.

*Effect of T cell responses on virus evolution.* To determine the association of virus evolution to SIV-specific T cell responses, the magnitude and breadth of the CD4+ and CD8+ T cell responses were monitored over the course of the study by T cell proliferation and gamma interferon ELISPOT, respectively (Figure 4.8). As expected, virus-specific CD4 and CD8 responses were undetectable in the "crash and burn" animal M11599 throughout infection. The ability to mount a T cell response during therapy varied widely among the other animals in the study. After treatment interruption, CD8+ T cell responses increased significantly in PMPA responders, a finding that confirms the maintenance of T cell memory responses in this group. Predictably, the poorest CD8+ response was observed in the responder animal infected with the most complex quasispecies (M10999). The differences in the magnitude of the T cell responses observed were not associated with a consistent change in the viral quasispecies, however.

Analysis of the breadth of the CD8+ T cell responses was further evaluated in the four Mamu-A*01 animals (2 poor responders and 2 responders; Figure 4.9). Although both poor responders and responders recognized little more than the immunodominant CM9 epitope (12) during therapy, the repertoire of recognized epitopes increased in the

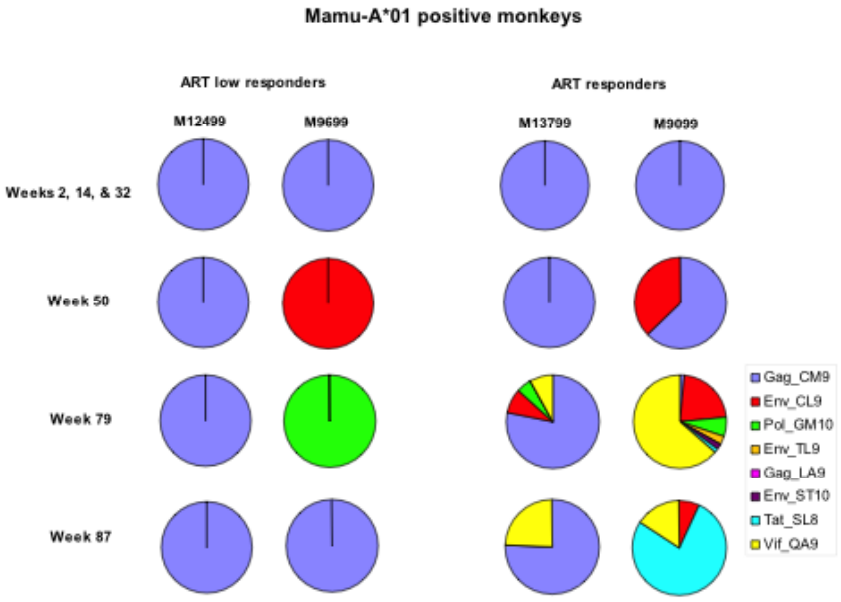



Figure 4.9. The breadth of the CD8+ T cell response in each Mamu-A*01 positive animals. Shown is the relative contribution of each of the 8 indicated Mamu-A*01 restricted epitopes to the total response measured during the treatment period (weeks 2, 14), and after PMPA was withdrawn (weeks 32, 50, 79, 87).

two Mamu-A*01 drug responders after treatment interruption. In one of these animals (M13799), the increase in the breadth of epitope recognition was associated with an increase in the viral quasispecies complexity. However, in the second animal (M9099), a shift in epitope recognition occurred without a detectable change in the quasispecies.

*Factors associated with response to PMPA.* It was clear from these studies that SIV/DeltaB670-infected monkeys differed with respect to their response to PMPA and, not surprisingly, a robust response to PMPA correlated with prolonged survival and maintenance of T cell function. Because treatment was initiated during acute infection with the same high dose stock in all of the animals, we sought to determine factors that were present prior to treatment that may have played a role in drug responsiveness. Although the parameters varied among the animals, no difference in virus burden (Figure 10a) or magnitude of SIV-specific CD4+ (Figure 10b) and CD8+ T cell responses (Figure 10c) that were present on the day therapy was initiated (2 weeks post-infection) were identified. However, the quasispecies expressed during the primary infection appeared less complex in responders than that observed in the poor responders, even though both groups were inoculated intravenously with the same stock (Figure 10d). Although the difference between the two groups fell short of statistical significance due to the small number of animals in each group ($P = 0.057$ Mann-Whitney U, two-tailed), these results imply that the virologic response to PMPA may be influenced by the degree of viral complexity that existed prior to treatment.

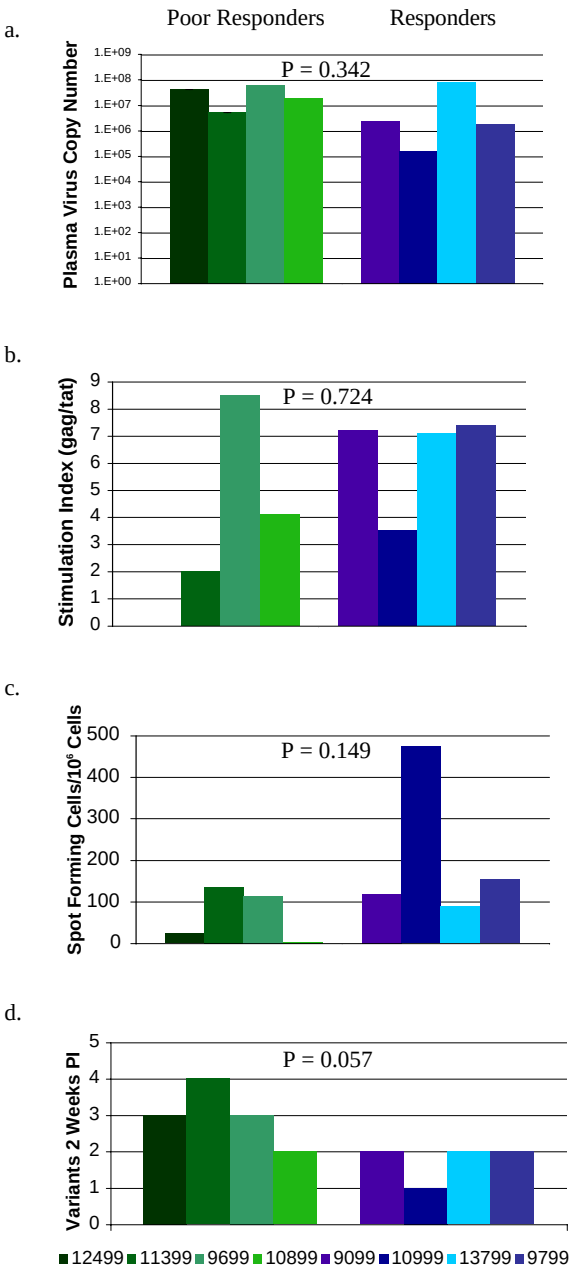



Figure 4.10. Comparisons of SIV-specific lymphoproliferative responses (a), CD8+ T cell responses (b), plasma virus copy number (c), and the number of viral variants (d) present prior to treatment. Individual values obtained at 2 weeks pi for poor responders (left most bars) and responders (right most bars) are shown. Statistical comparisons of the geometric mean values are reported in the figure as the P value. Statistical analysis of the proliferative response was calculated using 3 poor responders because results for M12499 were not available. The number of viral variants discernable 2 weeks pi was determined visually. Statistical analyses performed by Mann–Whitney U test, two-tailed analyses.

***Discussion*.**

Virus evolution was examined in SIV/DeltaB670-infected rhesus macaques undergoing antiretroviral therapy with PMPA. We focused on the V1-V2 region of *env* for these studies because it is the most hypervariable region in SIV (231) and has been highly informative in our hands in identifying the phenotypic properties of specific genotypes (16, 252, 287). Because all of the monkeys were inoculated intravenously with the same well characterized virus stock (16) and received identical treatment, this study provided a unique opportunity to characterize not only the virologic and immunologic parameters associated with drug responsiveness, it also enabled dissection of the impact of host adaptation and immune escape on this process. The latter studies are important because understanding how the virus responds to therapy at the molecular level would greatly aid in the design of adjunctive therapies that could enhance control of virus during treatment and promote continued control once treatment is discontinued. Host adaptation drives virus evolution independent of immune pressures by selecting for genotypes with greater replication fitness (and consequently, increased virulence; (150). Distinguishing between the two evolutionary pressures should provide important insight in evaluating drug efficacy because the ideal antiretroviral agent should not only reduce virus burden and thereby reduce the rate of immune escape, it should also suppress the emergence of more virulent genotypes. In our study, we were able to provide additional insight into host adaptation by identifying genotypes in the SIV/DeltaB670 quasispecies selected for by passage in human versus primate cells *in vitro*. A single passage of the primary stock of SIV/DeltaB670 in human cells resulted in a clonal infection with B670 clone 3. Conversely, B670 clone 12 was selected for by serial *in vitro* passage in monkey

PBMC. Not surprisingly, a common feature of the infection in untreated animals and poor responders was the universal loss of the B670 clone 3 genotype. Loss of this genotype as a result of poor replication fitness represents a unique example of viral divergence associated with adaptation to the host that is independent of immune escape.

Monkeys were divided into drug responders (>1 log reduction in virus burden) and poor responders (a reduction of $\leq$1 log) when compared to untreated infected controls. Serial HTA of virion cDNA revealed significant changes in expression of the SIV/DeltaB670 quasispecies associated with chronic infection and treatment. Virus infection in poor responders paralleled that of untreated controls, with the initial infection characterized by expression of a complex quasispecies that changed during disease progression. Virus evolution was readily apparent by the appearance and disappearance of new genotypes in both poor responders and untreated animals over time. These findings were not surprising because they were consistent with previous reports describing evolution driven by escape from B and T cell immunity known to occur in both SIV (11, 48) and HIV (155, 310) hosts. What has not yet been previously clear, however, is the contribution of host adaptation to this process.

Responders expressed a less complex quasispecies that remained more constant over the course of infection and treatment. These findings were most dramatically depicted in the three monkeys whose virus loads were reduced below the level of detection as a result of therapy. Importantly, not only was the emergence of novel genotypes less frequent, but the poorly fit "humanized" B670 clone 3 genotype remained as the dominant genotype expressed in two of these animals until the end of the study, over a year after treatment was stopped.

Regardless of the response to therapy, viral rebound was observed in all animals after treatment was stopped. Surprisingly, this event was not associated with the emergence of novel genotypes like that observed in some HIV+ patients during treatment interruption (53, 89). Cessation of HAART in these individuals, however, is usually accompanied by the emergence of drug sensitive virus with greater replication fitness than the drug-resistant predecessors. In our studies, PMPA resistance mutants remained fixed in the population in the poor responders, a condition that may not have allowed a clear window with which to observe these effects.

Changes in the V1-V2 sequence were also analyzed to determine whether the suppression in virus evolution observed in the responders affected sequences associated with antibody escape. V1-V2 sequence analysis reflected that observed by HTA, with the responder animal showing little change in this region during the course of the infection. Interestingly, the V1-V2 evolution observed in the poor responders and untreated controls did not consistently result in the acquisition of N-linked glycosylation sites. Rather, similar to the observations of Courgnaud et.al. (67) who examined adaptation of sooty mangabey propagated virus during passage in rhesus macaques, the V1 changes observed in our study appeared to have a greater impact on O-linked glycosylation sequences associated with a hotspot for insertions/deletions.

The emergence of the K65R/N69S/T *pol* mutations associated with PMPA resistance was also examined. As expected for the high rate of replication that persisted in the face of therapy, mutant genotypes readily emerged during the course of therapy in the poor responders. Interestingly, these mutations became fixed in the population in these animals and remained as the dominant genotype a year after treatment was

interrupted. In contrast, as expected by the slower rate of replication associated with drug responsiveness, this genotype was slow to emerge in the responder animal and was lost after treatment was discontinued.

An examination of the impact of SIV-specific T cell responses on virus evolution was also examined. No significant difference in either the magnitude or the breadth of the T cell response was observed in responders and poor responders during therapy. However, a significant change in these responses became apparent in the responders after treatment was stopped. Interestingly, the increase in the breadth of the epitope recognition observed in one of the animals was not associated with a visible change in the viral quasispecies. Although the V1-V2 region was not directly involved in the T cell epitopes recognized in this animal, one would predict that effective T cell-mediated control of replicating virus would select for escape variants that would be identified by their own V1-V2 sequence. These data suggest that changes in the breadth of the T cell response do not always parallel that of virus evolution and that accurate assessment of a functional role of T cell immunity should include not only analysis of the T epitope responses but analysis of the viral sequence encoding these epitopes as well. Our findings are nevertheless consistent with previous observations in that virologic control associated with drug therapy, in general, coincides with the ability to maintain a virus-specific T cell response (108, 177, 301). Further, effective therapy can have a long-term impact on the maintenance of the host's T cell immunity even after it is withdrawn, and support the hypothesis that an effective means of immune evasion is rapid evolution.

The dramatically different response to PMPA observed among the animals prompted us to examine both immunological and virological parameters that were present

prior to the initiation of therapy to identify factors that would be predictive of treatment responsiveness. Clearly, this knowledge would significantly aid in refining treatment of HIV-infection in humans. Interestingly, there was no association between PMPA responsiveness and either the peak virus loads or the magnitude of the T cell response prior to therapy. However, animals that responded well to therapy expressed a less complex quasispecies during peak viremia than those who did not. These findings are consistent with the recent observation in HIV+ patients where it was determined that expression of fewer genotypes during therapy correlated with the ability to control rebound upon interruption of HAART (145). Using the monkey model where infection could be controlled, our data further suggests that a pattern of less complexity *before* therapy may be predictive of virological control *during* therapy. These results imply that intrinsic factors that exist in the host at the time of the initial infection may play a role in restricting initial infection with multiple genotypes, which may not only provide a superior environment for the induction of T cell immunity, but also assist in the control of virus by antiretroviral drugs. Together, these results are encouraging because they support the concept that therapeutic immunization may prove beneficial in augmenting viral control in drug responders because the target for protective immunity is less variable.

# Chapter 5. Virus Evolution in SIV Infected Animals Given Therapeutic DNA Immunizations During Transient Antiretroviral Drug Treatment.

***Introduction.***

An estimated 40 million people are infected with the human immunodeficiency virus (HIV)(239), Currently, treatment of HIV infected people relies on a cocktail of antiretroviral drugs (highly active antiretroviral therapy, HAART) targeting different viral enzymes. These therapies are unable to eradicate the viral reservoir (54) and over time treatment fatigue and/or the emergence of drug resistant viral variants occurs (20, 64). This frequently heralds an increase in virus burden and a return of symptoms. Therefore, strategies need to be developed that augment the effects of therapy and control virus replication when therapy is withdrawn. In the nonhuman primate model of HIV infection, live attenuated vaccines have provided protection from infection (68, 70, 222). However, safety concerns have prevented their development for use in humans (18, 255). DNA vaccines offer a safe alternative while duplicating many aspects of natural infection and live attenuated vaccines. Using gene gun technology, the DNA is deposited intracellularly (92, 314) and is subsequent transcribed and translated into proteins. The endogenous production of antigen results in a protein with native posttranslational modification, conformation, and oligomerization (292) and generates antigen specific immune responses (87). DNA vaccines have provided protection from disease progression (15, 25, 37, 147, 246), and persistent infection against a heterologous mucosal SIV challenge (101). Additionally, a therapeutic DNA vaccination administered during antiretroviral therapy (ART) successfully increased the virus specific immune response and protected against viral rebound after drug was withdrawn (102), demonstrating the benefits of vaccinating during drug therapy as a means to improve control of viral replication. What remains unknown is the effect of ART and

immunization on the expressed viral genotypes circulating during treatment and/or after its withdrawal. This understanding may provide a framework to aid in the design of new strategies to improve control of viral replication via augmenting the virus specific immune response during drug therapy.

Virus evolution limits the effectiveness of the host's immune response by constantly changing the viral targets the response is generated against. One strategy to limit the ability of a virus to escape immune recognition is to halt, or reduce viral replication to allow the immune response to keep pace. To analyze the effects of therapeutic interventions on virus evolution, we monitored the genotypes expressed in plasma from animals given DNA immunizations during ART (102). The goal of the DNA immunizations was to enhance immune responses primed by the infection or induce *de novo* responses to previously unrecognized targets, thereby broadening the response. The study compared DNA vaccines encoding SIV Gag and Tat proteins (*gag/tat*) alone or in combination with 19 CTL epitopes (*gag/tat*/multi-epitope) to further enhance the CD8+ T cell response against the virus. The rational behind the study was to decrease virus replication with PMPA while enhancing/generating a virus specific immune response of sufficient magnitude or breadth to control the virus once drug was withdrawn. Sixteen animals were inoculated intravenously with SIV/DeltaB670 and treated daily for 28 weeks with the reverse transcriptase inhibitor (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA). PMPA was initiated two weeks post inoculation (pi) and six therapeutic vaccinations were administered via gene gun at four-week intervals starting six weeks pi. Virologic and immunological parameters were compared between immunized animals and control animals treated with PMPA alone.

After the initiation of PMPA, the animals demonstrated either a drug responder or poor responder phenotype. A drug responder was defined as an animal that maintained a virus load at or below $10^4$ viral RNA copies /ml plasma during the period of ART. There was no significant difference in the virus loads measured two weeks post inoculation demonstrating that the level of virus replication when ART was initiated did not influence the subsequent response to PMPA. The plasma virus burden and immune responses (lymphoproliferation and CD8+ T cell responses against specific epitopes or peptide pools) in vaccinated animals that failed to respond to ART were similar to unvaccinated controls. In contrast, vaccinated animals that responded to PMPA demonstrated significantly higher LPR and CD8+ T cell responses than unvaccinated controls that responded to ART. Therapeutic immunizations with the *gag/tat*/multi-epitope DNA vaccine proved to be efficacious ($P = 0.029$, Fishers exact) in containing viral replication for six months after treatment withdrawal in responder animals. Control of virus replication correlated with maintenance of CD4+ cell counts, higher virus-specific lymphoproliferative responses, and an increased magnitude and breadth in the CD8+ T cell response.

As a first step towards determining the effects of therapeutic vaccination and transient drug treatment on virus evolution, we monitored virus evolution in these animals by heteroduplex tracking analysis (HTA). The outcome of the study suggested that responder animals given the *gag/tat*/multi-epitope immunizations would demonstrate the least virus evolution when compared to either immunized poor responders or control animals given drug treatment alone.

*Materials and Methods*

**Animals, viral challenge, and anti-retroviral therapy.** The animals, viral challenge and antiretroviral therapy used in this study have been previously reported (102).

**DNA vaccines and immunizations.** The DNA vaccine design and immunizations have been reported previously . Briefly, the multi-epitope DNA vaccine was prepared by inserting 21 SIVmac239 CTL epitopes (10) into a cocktail of nine hepatitis B core antigen (HBcAg) carrier expression vectors (PowderJect Vaccines, Inc. Middleton, WI). Each HBcAg carrier plasmid contained 1-3 epitopes with expression driven by the CMV immediate early (IE) promoter. The *gag/tat* DNA vaccine consisted of a combination of two plasmids, each encoding either the full-length SIVmac239 *gag* or *tat* gene down stream of the CMV IE promoter. The plasmid DNA was individually precipitated onto gold particles and mixed before administration. Sedated animals received a total of 2 μg of plasmid DNA via gene gun administered in at 16 skin delivery sites that included the area over the abdominal and inguinal lymph nodes. DNA vaccination was initiated six weeks post inoculation (4 weeks post therapy initiation). The immunizations were repeated every four weeks: resulting in a total of six vaccinations during drug treatment.

**Plasma virus load and heteroduplex tracking analysis (HTA).** Quantification of the plasma virus burden and HTA have been previously reported (102).

***Results.***

*Study design*. The effects of therapeutic vaccination on virus evolution in drug treated SIV infected animals was explored by heteroduplex tracking analyses (HTA) of the viral genotypes present in the peripheral blood of immunized infected animals followed by a comparison to a similar analysis of infected animals that received drug therapy alone. The study design and anticipated outcome of PMPA on plasma virus burden is shown in Figure 5.1.

The plasma virus loads in all of the animals reached a peak two weeks post infection and then decreased due to the immune response induced by infection and/or vaccinations. In untreated animals, those given PMPA alone and immunized poor responders the virus burden remained above $10^5$ virus copies/ml plasma throughout the study period. The plasma virus burden in some of the responder animals (immunized or ART treated alone) was maintained below $10^4$ copies/ml plasma but remained detectable throughout the treatment period. After treatment withdrawal the plasma virus burden increased to or above $10^4$ until the studies conclusion. The plasma virus load was undetectable in animals with the best response to either ART alone or immunizations during ART. After treatment withdrawal the virus burden generally remained below $10^4$.

We performed HTA using plasma cDNA because this represents the genotypes actively engaged in the infection whereas proviral DNA genotypes in PBMC are confounded by archival sequences that may not be actively replicating. The use of viral RNA as the starting template for this analysis, and the knowledge of the genotypes comprising the challenge stock (287), allowed us to make that discrimination.

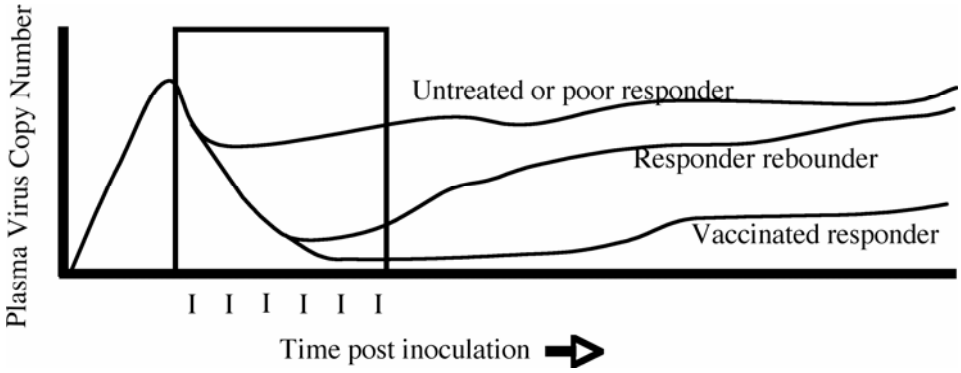



Figure 5.1 Study design with possible viral load outcomes for PMPA treatment with and without DNA vaccination. X axis is time post infection, Y axis is virus copy number/ml plasma. I represents 6 post infection DNA vaccinations. Boxed area represents treatment period (weeks 2 through 30 pi).

*Clinical disease course.* The clinical disease course of the animals given therapeutic vaccination during antiretroviral drug therapy is summarized in Table 2. The animals were grouped as drug responders or poor responders based on maintenance of a plasma virus burden at or below $10^4$ during ART (Figure 5.2). Five animals that responded poorly to treatment (5/7) succumbed to infection with an AIDS defining illnesses within 465 days after inoculation. This is in contrast to animals that responded to treatment where all of the animals lived disease free until the study's end (sacrifices scheduled from 589 - 664 days pi). To determine if therapeutic immunizations prolonged survival in drug treated animals, we compared the length of survival from animals given the *gag/tat* multi-epitope immunizations or *gag/tat* alone, to the animals treated with PMPA alone and found there was no significant difference in survival (P= 0.442, and 0.505 respectively, Mann-Whitney U, two-tailed). In contrast, a comparison of all

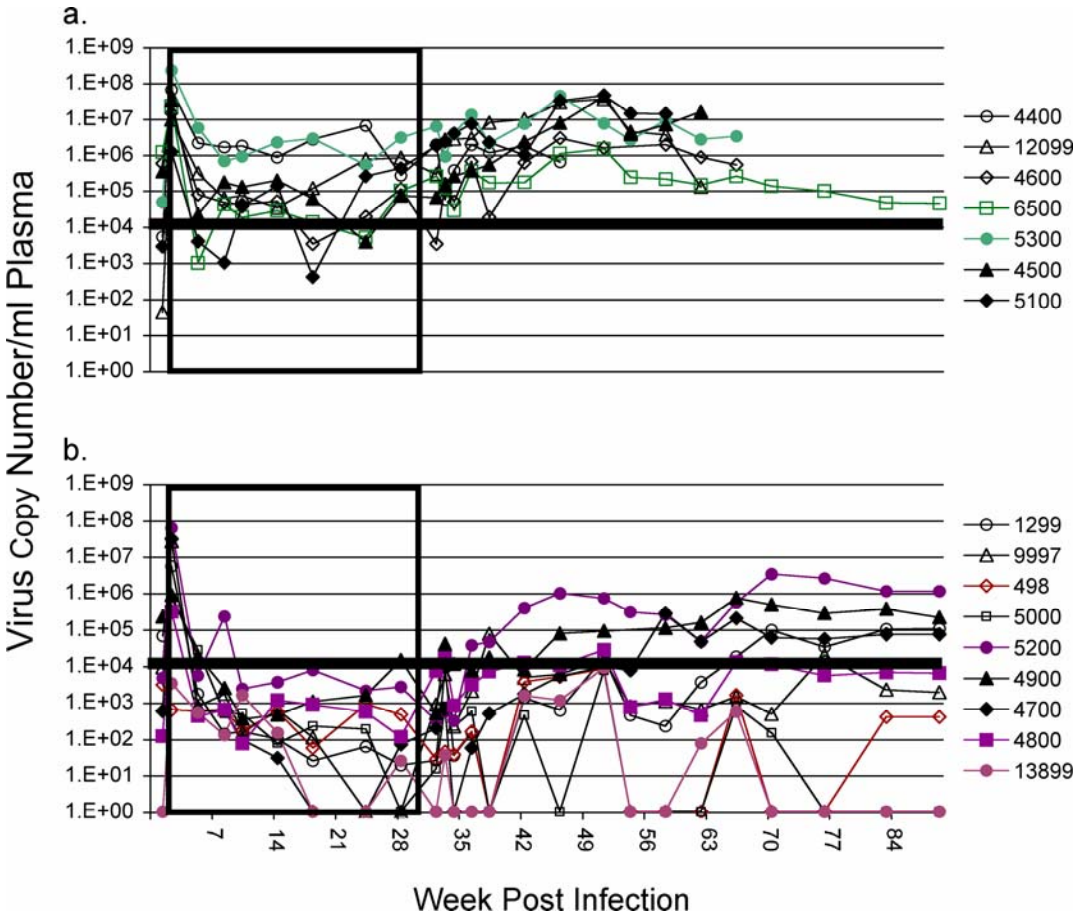



Figure 5.2. Plasma virus load in therapeutically immunized (A) poor responders and (B) responders animals over time post inoculation. The vertical line represents the average plasma virus burden from LTNPs ($10^4$ copies/ml). Plasma was serially sampled and virus copy number/ml plasma was calculated by real-time RT-PCR. Cutoff for the assay was 10 copies/ml. Boxed area represents treatment period (weeks 2-30 post infection). Open symbols represent animals that received the *gag/tat*/multi-epitope vaccinations and closed symbols represent animals that received the *gag/tat* vaccinations.

TABLE 2. Study Outcome of Therapeutic DNA Immunizations[a] SIV/DeltaB670 Infected PMPA Treated[b] Animals

| Group | Animal Number | Survival (Days) | Mamu Haplotype | Cause of Death |
|---|---|---|---|---|
| *gag/tat*/multi-epitope PMPA poor responder | M4600 | 465 | A*01, B*17 | Lymphoproliferative disease |
| | M4400 | 319 | A*01 | Enterocolitis, meningitis |
| | M12099 | 407 | A*01, B*03 | Enterocolitis, Pneumocystis pneumonia, bacterial pneumonia |
| | M6500 | 649[c] | A*01, A*08, B*03 | Euthanasia |
| *gag/tat* PMPA poor responder | M5100 | 407 | A*02, A*08, B*01, B*03 | Lymphosarcoma, pneumonia |
| | M5300 | 527 | A*02, B*01 | Lymphosarcoma |
| | M4500 | 451 | A*02, A*08, B*03 | Bacterial septicemia, lymphosarcoma |
| *gag/tat*/multi-epitope PMPA responder | M5000 | 649[c] | A*01, B01 | Euthanasia |
| | M9997 | 634[c] | A*01, A*02, B*01 | Euthanasia |
| | M1299 | 647[c] | A*01, B*03, B*17 | Euthanasia |
| | M498 | 647[c] | A*01, A*08, B*03 | Euthanasia |
| *gag/tat* PMPA responder | M4900 | 636[c] | A*08, B*03, B*17 | Euthanasia |
| | M4700 | 653[c] | A*02 | Euthanasia |
| | M4800 | 655[c] | A*02, A*08, B*03 | Euthanasia |
| | M5200 | 655[c] | A*02, B*01 | Euthanasia |
| | M13899 | 653[c] | B*03 | Euthanasia |

[a] Six therapeutic DNA vaccinations were given starting 6 weeks pi and repeated at 4 week intervals.
[b] Daily PMPA treatment (20 mg/kg) initiated 14 days pi, continued six months then withdrawn.
[c] Scheduled sacrifice

animals that responded to therapy (regardless of immunization status) to those that responded poorly showed a significant difference in the length of survival (P = 0.004,Mann-Whitney U, two tailed). To determine if the addition of the multi-epitope vaccine prolonged survival, we compared the length of survival between animals that received this preparation and those that received *gag/tat* alone and found no difference in survival (P = 0.279, Mann-Whitney U, two-tailed). This result could have been due to

termination of the study before infection had reached its natural conclusion, however. The plasma virus loads in the majority of the responder animals given the additional multi-epitope preparation was below 2 X $10^3$ when the experiment was terminated while the majority of responders animals given only *gag/tat* immunizations was above 7 X $10^4$. This difference is not a significant however (P = 0.286, Mann-Whitney U, two-tailed).

*Viral genetic evolution* in vivo. To determine if we could discern a difference in viral evolution by HTA between drug treated animals given therapeutic immunizations and those given drug alone we compared their HTA patterns. At acute infection, prior to the initiation of therapy, we did not detect a difference in the expressed genotypes between animals that received immunizations (Figures 5.3 and 5.4) and those that did not (Chapter 4, Figures 4.5 and 4.6), regardless of their subsequent response to PMPA. All of the animals expressed a minimum of at least one of the two genotypes dominant in an inoculum during acute infection.

**Poor Responders**. Early after treatment initiation (week 2 - 10 pi), immunized animals that responded poorly to PMPA maintained expression of the genotypes dominating the acute infection, regardless of which immunizations they received (Figure 5.1). Throughout the remainder of the treatment period, HTA revealed a complex pattern of genotypes expressed that changed over the study period, like that seen in the drug treated controls (Chapter 4, Figure 4.5). Two of the animals given drug treatment alone (Chapter 4, Figure 4.5, M9699 and M11399) and three vaccinated animals (Figure 5.3, M5300, M4500, andM4600) maintained expression of the same genotypes expressed at the end of the treatment period after treatment was withdrawn. The control animals maintained this pattern of expression approximately 12 weeks whereas those given

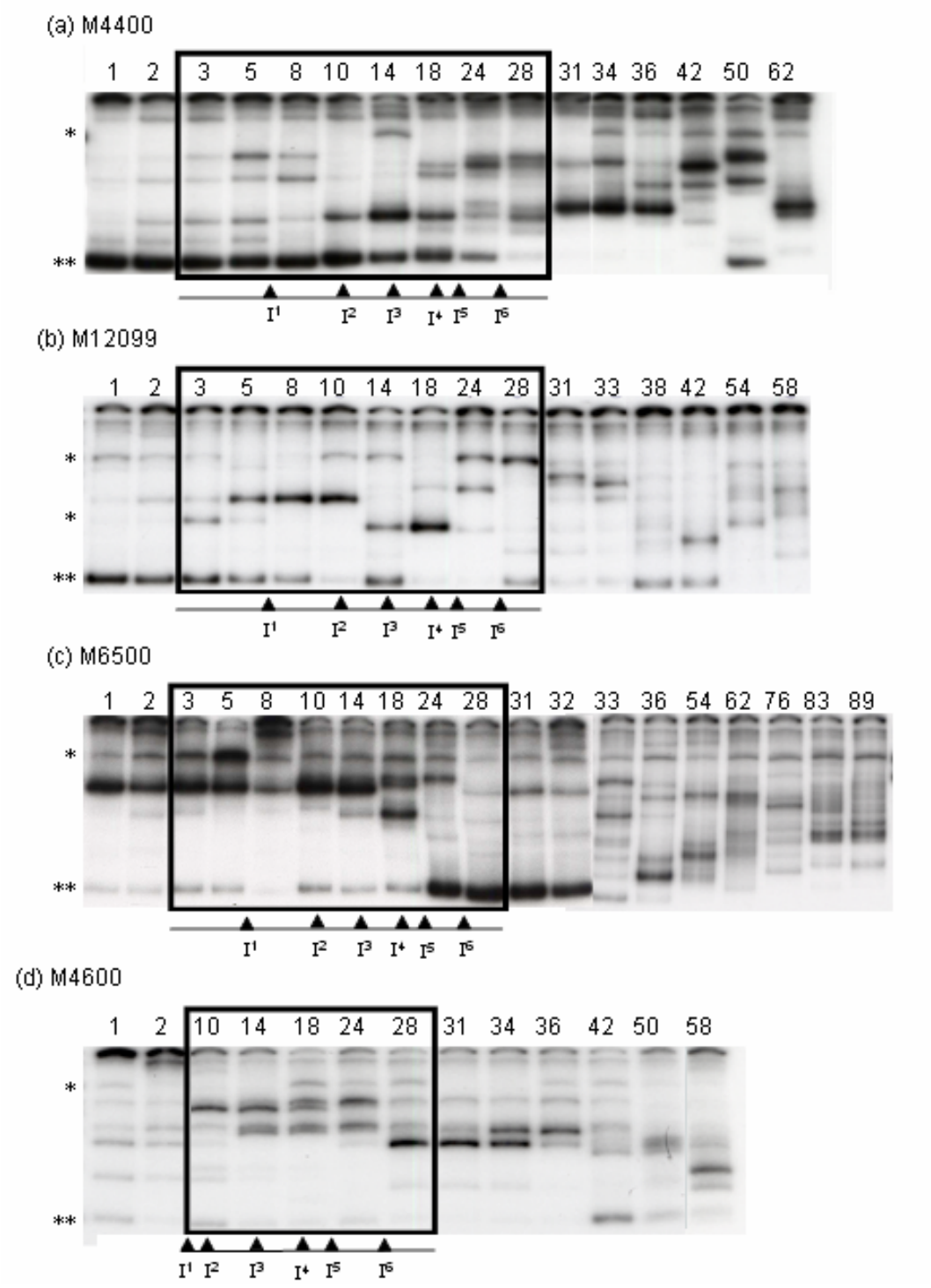



Figure 5.3. Continued

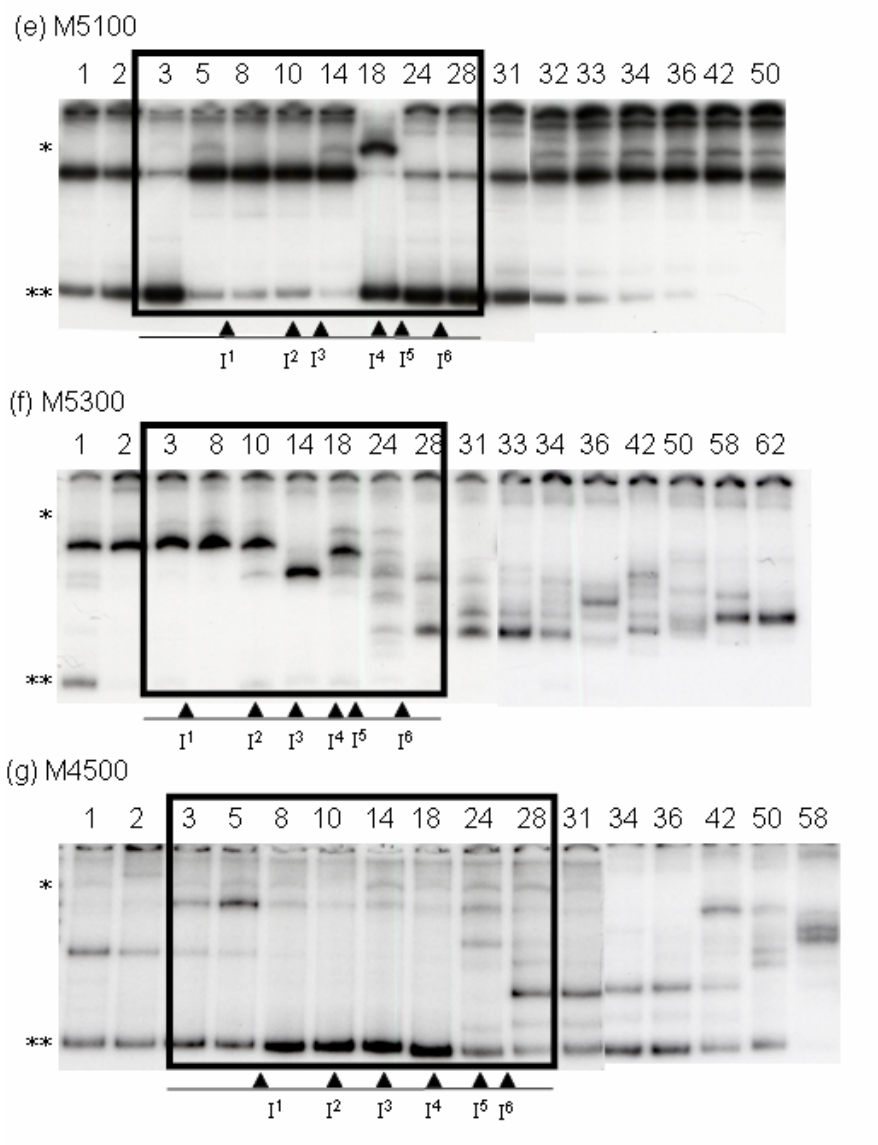



Figure 5.3. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated and therapeutically immunized poor drug responder macaques. Numbers across the top indicate week post inoculation. Daily PMPA treatment was initiated 2 weeks pi and continued for 28 weeks. Boxed area represents samples collected during therapy. The last week is the necropsy sample. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. (a) M440, (b) M12099, (c) M6500, (d) M4600 (e) M5100, (f) M5300, (g) M4500.

immunizations maintained them for approximately six weeks, however. This truncation of expression suggests that vaccine induced virus specific immune responses may have influenced virus evolution/selection. After treatment was withdrawn however, the majority of the immunized poor responders animals exhibited ongoing viral evolution with the emergence of more variants over time.

**Responders**. In animals that responded to PMPA, the vaccinated animals expressed more viral genotypes up to week 10 pi than animals given PMPA alone (Figures 5.4 and 4.6). At this point, the vaccinated animals had received a single immunization. This result may indicate the DNA immunization had driven a more robust evolution of the virus population. After week 10 pi, we were unable to complete the comparison during the remainder of the treatment period because we could not reliably amplify cDNA from the majority of the responder animals (Figure 5.4). After treatment withdrawal, we were again unable to track virus evolution in the majority of the animals that received the *gag/tat*/multi-epitope immunizations because they did not rebound and thus their virus loads were below our detection limit. This result indicates the immunization protocol proved beneficial in preventing virus rebounded when compared to animals given ART alone. The HTA pattern from the remaining immunized responder animals duplicated that seen in animals given drug treatment alone, with expression of a less complex quasispecies in the majority of these animals.

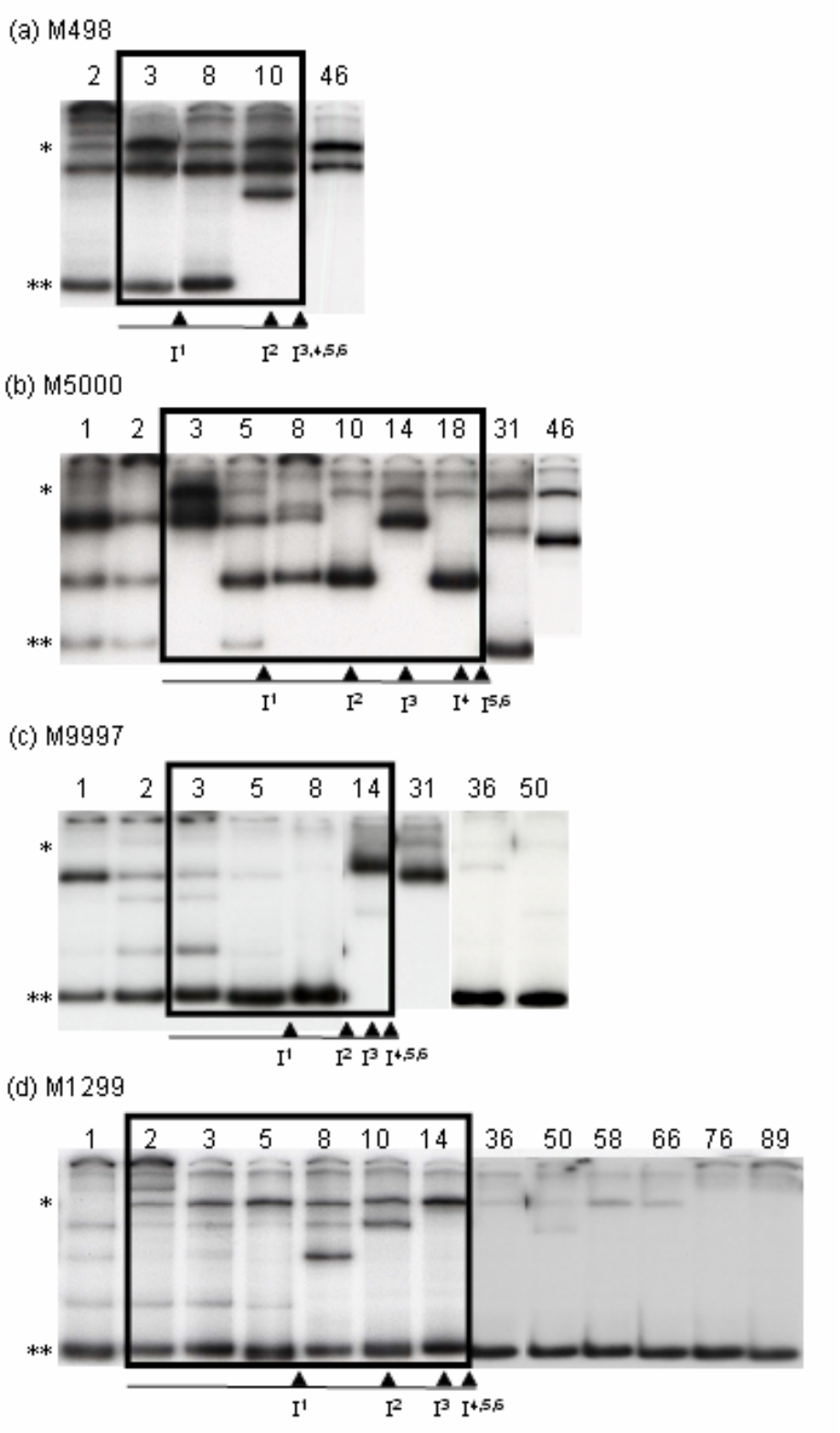



Figure 5.4. Continued.

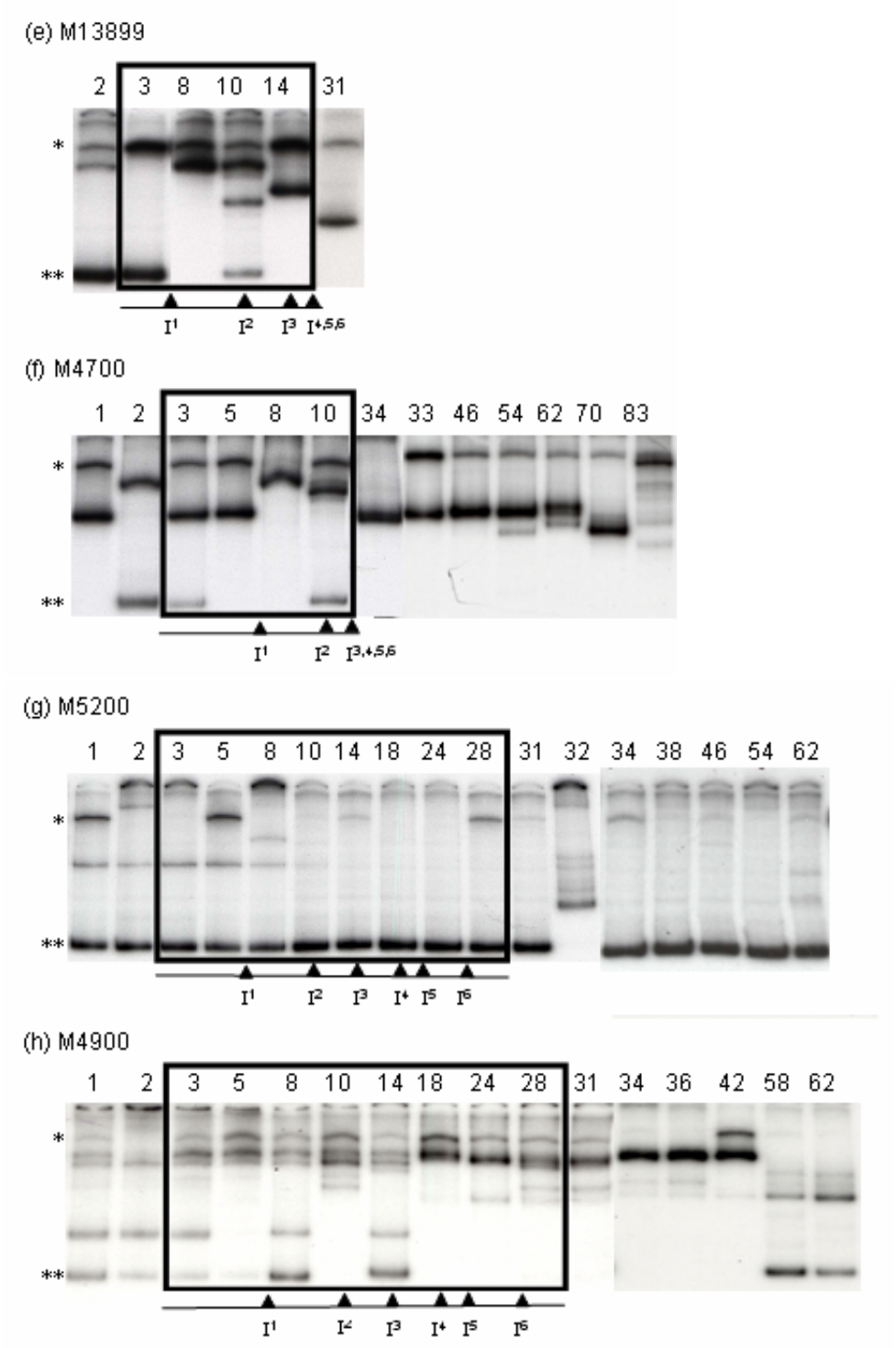



Figure 5.4. Continued.

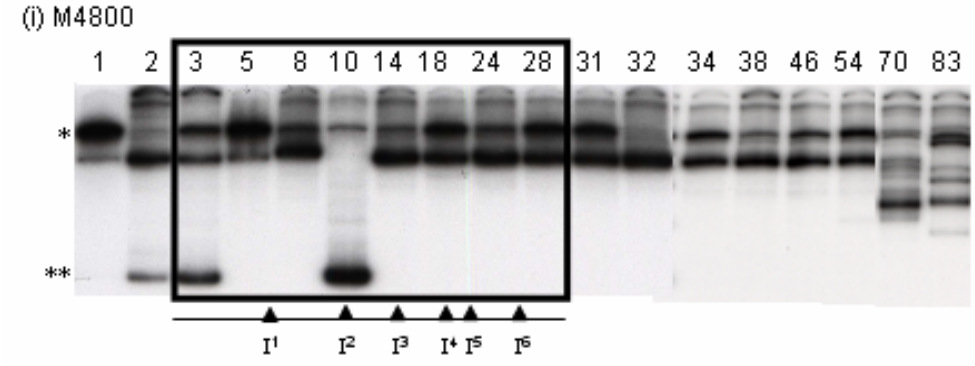



Figure 5.4. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated and therapeutically immunized drug responder macaques. Numbers along the bottom indicate week post inoculation. Daily PMPA treatment was initiated 2 weeks pi and continued for 28 weeks. Boxed area represents samples collected during therapy. The last week is the necropsy sample or the latest available with virus burden within detection limits. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. (a) M498, (b) M5000, (c) M9997, (d) M1299 (e) M13899, (f) M4700, (g) M5200, (h) M4900, (i) M4800.

***Discussion.***

We monitored virus evolution in SIV infected animals given therapeutic DNA immunizations with either gag and tat whole genes or gag/tat plus a multi-epitope DNA vaccines during a period of antiretroviral therapy (102). The V1-V2 region of the envelope gene was used for these studies because it is the most hypervariable region of the genome (231) and has been used to identify the genotypes present in the virus isolate used in this study, SIV/DeltaB670 (16, 252, 287). Like the PMPA treated controls (Chapter 4), animals that received therapeutic immunizations were divided into drug responder or poor responder phenotypes based on maintanance during ART of a plasma virus burden either above or below $10^4$ viral copy numbers/ml. The most efficacious vaccine was the *gag/tat*/multi-epitope DNA preparation. A therapeutic effect was seen only in the PMPA responders however.

We had anticipated that animals that responded to PMPA that received therapeutic immunizations would have had less evidence of virus evolution after treatment withdrawal than animals given drug treatment alone. This was based on the finding that animals that received PMPA alone were unable to control virus replication after treatment withdrawal to the same degree as that observed in the immunized responder animals (102). Since virus evolution is driven in part by virus replication (128, 204, 311, 326), we reasoned that low levels of virus replication would translate into less evolution of the virus population. Furthermore, virus specific T cell responses have also been shown to be associated with greater control of virus replication (35, 142, 158, 193, 256, 307). Thus, inclusion of a vaccine during the generation of the virus specifc T cell response, and during a period of drug treatment that can potentially aid in reducing virus

replication, should reduce viral evolution further than that seen in animals given ART alone. This hypothesis was supported by the finding that animals that received the *gag/tat*/multi-epitope immunizations maintained their CD4+ T cell counts and virus-specific T cell responses while those treated with ART alone did not.

Evaluation of virus evolution by HTA revealed that the therapeutically immunized animals mirrored what was seen in the drug treated control animals, with poor responders displaying a complex pattern of genotypes and responders demonstrating less evolution of the viral quasispecies. Our inability to identify less evolution of the virus population in animals responding well to the vaccine could be due to several reasons.

1 - The vaccine was not potent enough to induce global changes in the population.The initial reports of therapeutic immunizations of HIV infected humans using various vaccine strategies demonstrated that although virus specific T cell responses were induced by immunization, immunotherapy failed to reduce the plasma virus burden (32, 110, 160, 209, 304). These strategies were tested without the advantage of ART however, in an attempt to allow an enhanced/expanded immune response to develop. More recently, an HIV vaccine trial used as an adjunct to HAART demonstrated control of transient increases in viral rebound after treatment withdrawal. Although there was no change in the T cell counts (183), eight of 13 vaccinees demonstrated virus-specific IFN-γ ELIspot responses. In the SIV infected macaque, studies using different vaccines given during ART produced results similar to our study, with improved control of virus replication after treatment withdrawal/interruption accompanied by enhanced virus specific T cell responses (125, 178, 289). Allen et al. (11) demonstrated that an immunodominant Tat specific CTL response rapidly selected

for viral variants with a mutation in Tat with few other sequence changes throughout the genome. The authors reasoned that the CTL response was able to eliminate infected cells expressing the wild-type sequence because they were unable to detect the wild-type sequences in the animals examined. An increase in the magnitude and breadth of the T cell response was clearly demonstrated in the animals that received therapeutic immunizations in our study (102). The broadening of the immune response induced by immunizations, and the high degree of similarity between the vaccine and challenge strains, would suggest that more epitopes would be targeted by the CTL response in immunized animals, and result in elimination of infected cells expressing these epitopes. The net effect of this phenomenon could result in a global change in the population and therefore a loss of particular V1-V2 sequences.

2– Monitoring V1-V2 will not demonstrate virus evolution driven by immune evasion because it does not encode T cell epitopes. Alternatively, the Allen et al. (11) study also demonstrated that escape from a CTL response in one epitope resulted in retention of the wild-type sequences in other regions of the genome. If this occurred in our animals and the CTL response did not eliminate infected cells expressing the mutated epitope, HTA would not reveal evolution of the virus population unless individual epitopes were being directly monitored. The reason for our findings are unknown at present and must await a more thorough evaluation of T cell epitope sequences during therapy.

3– The most important animals for monitoring these events had virus loads too low to analyze. Mutations within the viral genome can result in a virus either an advanced (72, 169, 263, 264) or a delayed disease progression phenotype (151, 165, 200).

Identifying sequence changes that contribute these phenomena would aid in the design of antiviral therapies. The plasma virus burdens in some of the animals were so low that these samples could not be included in our analysis. This suggests that these animals could have been infected with an attenuated viral quasispecies present in the inoculum. This result is unlikely because infection of over 500 animals with this SIV isolate has resulted in disease progression in over 95% of the animals (M. Murphey-Corb, personal communication). Instead, the viral variants infecting these animals could have been attenuated after infection. Multiple immune and non-immune host factors have been identified that affect virus replication/disease host factors (34, 47, 182, 202, 230, 282). Analysis of the viral genotypes infecting these animals could lend insight into viral factors associated with non-progression.

In summary, selective pressures imposed on the viral genotypes after transmission into a new host result in the generation of a broad quasispecies with potentially different pathogenic potentials. The additive effects of adjunctive drug treatment alone, when effective, can delay/prevent these events. Further studies are needed, however, to fully define these events.

# Chapter 6. The Effects of DNA Vaccination Pre and Post Inoculation on Virus Evolution in SIV Infected Drug Treated Animals.

***Introduction.***

Approximately 11,000 people are infected with HIV daily (294) and the current treatments are unable to provide long-term control of infection to those with access to the drugs. These facts clearly highlight the need for an efficacious vaccine. There are numerous vaccine trials underway with the goal of inducing virus specific T cell responses against HIV (one resource for information is (http://www.hvtn.org/science/trials.html). These vaccines may fail to provide sterilizing protection against infection. Whether participants will be at a selective after infection is currently unknown.

In this chapter we address the role of vaccine induced priming of the SIV specific immune response in augmenting the control of virus during post inoculation ART and immunotherapy. The antiviral treatments have been presented previously (Chapter 5). Vaccine primed animals demonstrated either drug responder or poor responder phenotypes, like that observed in the other groups (Chapters 4 and 5). Again, there was no significant difference in the virus loads measured two weeks post inoculation, demonstrating prior immunization had no suppressive effect on the acute virus loads nor did it affect drug responsiveness. The plasma virus burden and immune responses (lymphoproliferation and CD8+ T cell epitope recognition) in vaccine primed animals that failed to respond to ART were similar to unvaccinated controls. In contrast, vaccine primed animals that responded to PMPA demonstrated significantly higher LPR and CD8+ T cell responses then unvaccinated controls that responded to ART. DNA immunizations delivered both pre and post inoculation with the *gag/tat*/multi-epitope DNA vaccine proved to be efficacious ($P = 0.015$, Fishers exact) in containing viral

replication for six months after treatment withdrawal and retention of CD4+ cell counts for one-year post treatment in responder animals. Control of virus replication correlated with maintenance of CD4+ cell counts, higher virus-specific lymphoproliferation, and an increased magnitude and breadth in the CD8+ T cell response.

We hypothesized that vaccine priming, in addition to maintaining CD4+ T cells, and increasing the strength and breadth of the T cell responses, would promote less evolution than that seen in animals given only therapeutic immunizations. To determine these effects, we monitored the V1-V2 region of the envelope gene from virus expressed in plasma throughout the study period in animals vaccinated pre and post inoculation and compared these results to animals given only therapeutic immunizations or drug treatment alone.

***Materials and Methods***.

**Animals, DNA vaccine preparation and protocol, and drug treatment**. The animals, vaccine, and drug treatment have been reported (Chapter 5).

**Heteroduplex tracking analysis (HTA).** The cDNA preparation and HTA protocol have been reported (Chapter 4).

***Pol* PCR amplification, sequencing and sequence analysis.** The amplification, sequencing and sequence analysis have been reported (Chapter 4).

***Results.***

*Study design*. The effects of DNA vaccinations pre and post infection on virus evolution in drug treated SIV infected animals was monitored throughout infection using a heteroduplex tracking analyses (HTA) of the viral genotypes present in the peripheral blood followed by a comparison to a similar analysis of infected animals that received either therapeutic immunizations during ART or drug therapy alone. The study design and anticipated outcome of PMPA and immunizations on plasma virus burden is shown in Figure 6.1.

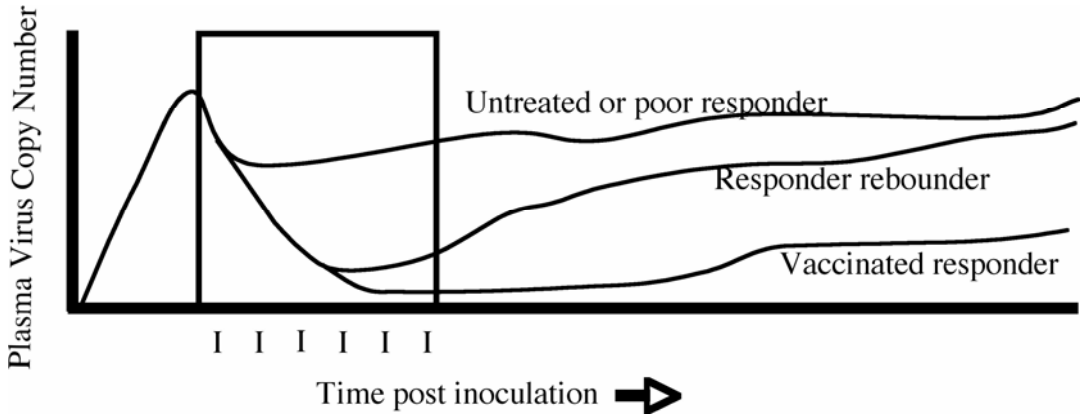



Figure 6.1 Study design with possible viral load outcomes for PMPA treatment with and without DNA vaccination. X axis is time post infection, Y axis is virus copy number/ml plasma. Large arrow represents 4 pre infection DNA vaccinations, I represents 6 post infection DNA vaccinations. Boxed area represents treatment period (weeks 2 through 30 pi).

*Clinical disease course.* The clinical disease course in animals that received DNA immunizations both pre and post infection is summarized in Table 3. Eleven animals were drug responders and five were poor responders (Figure 6.1). The animals that responded to treatment lived significantly longer than animals that responded poorly to treatment (P = 0.038, Mann-Whitney U, two-tailed). To determine if immunizations received both pre and post infection and response to treatment extended survival, we compared these animals to those immunized only therapeutically. We found there was no difference in survival within 16 months post-ART when comparing either the responders, or poor responders (P = 0.17, and 1 respectively, Mann-Whitney U test, two tailed). We additionally compared vaccine primed animals to those given PMPA alone and found there was no difference in survival when comparing responders and poor responders (P = 0.34, and 0.91 respectively, Mann-Whitney U test, two tailed). This result could have been due to termination of the study before infection had reached its natural conclusion. The geometric mean plasma virus burden in responders given PMPA alone was above 1 X $10^5$ while in vaccinated animals it was between 1 X $10^2$ – $10^3$. This indicates a loss of control of virus replication in the animals given drug treatment alone not seen in the vaccine primed animals.

TABLE 3. Study Outcome of SIV/DeltaB670 Infected PMPA Treated [b] Animals DNA Vaccinated[a] Pre and Post Inoculation

| Group | Animal Number | Survival (Days) | Mamu Haplotype | Cause of Death |
|---|---|---|---|---|
| *gag/tat*/multi-epitope PMPA poor responder | M10299 | 671[c] | A*01, A*02 | Euthanasia |
| *gag/tat* PMPA poor responder | M13299 | 291 | A*02, B*03 | Lymphoproliferative disease |
| | M12299 | 635[c] | A*08, B*03 | Euthanasia |
| | M12699 | 291 | B*17 | Mesenchymal proliferative disorder suggestive of SRV-D[e] |
| | M10297 | 465 | B*03 | Retroperitoneal fibromatosis consistent w/ SRV-D[e] |
| *gag/tat*/multi-epitope PMPA responder | M11197 | 671[c] | A*01, A*02 | Euthanasia |
| | M14199 | 678[c] | A*01, B*01 | Euthanasia |
| | M12899 | 676[c] | A*01, A*11, B*01 | Euthanasia |
| | M11499 | 669[c] | A*01, A*08 | Euthanasia |
| | M13999 | 678[c] | A*01, A*08, B*03 | Euthanasia |
| | M10497 | 676[c] | A*01 | Euthanasia |
| | M12997 | 669[c] | A*01 | Euthanasia |
| *gag/tat* PMPA responder | M11999 | 635[c] | Unknown[d] | Euthanasia |
| | M12599 | 637[c] | A*02 | Euthanasia |
| | M11299 | 635[c] | Unknown[d] | Euthanasia |
| | M13199 | 637[c] | Unknown[d] | Euthanasia |

[a] Four DNA vaccinations were given at 6-8 week intervals before inoculation and repeated at 4 week intervals starting 6 weeks pi.
[b] Daily PMPA treatment (20 mg/kg) initiated 14 days pi, continued six months then withdrawn.
[c] Scheduled sacrifice
[d] Haplotype was not Mamu; A*01, A*02, A*08, A*11, B*01, B*03, B*04, B*17
[e] All animals are SRV-D antibody negative prior to entry into the facility.

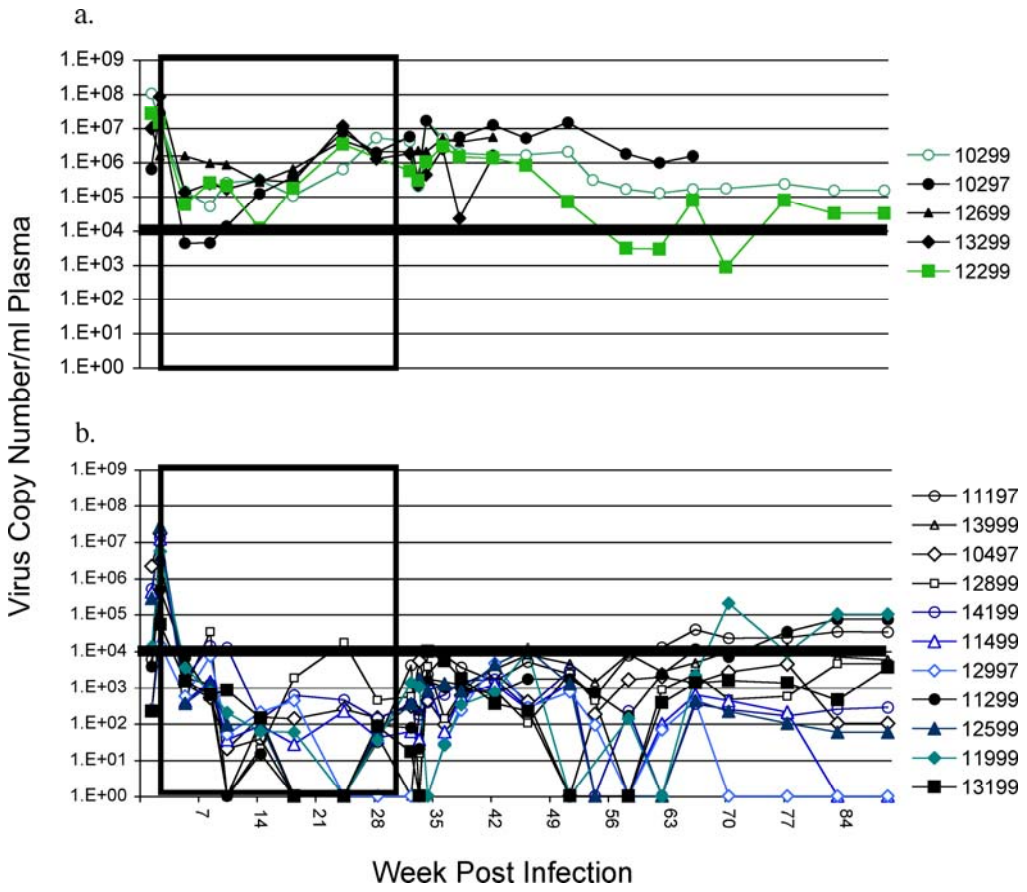



Figure 6.2. Plasma virus load in (A) poor responders and (B) responders animals immunized pre and post inoculation over time post infection. The vertical line represents the average plasma virus burden from LTNPs ($10^4$ copies/ml). Plasma was serially sampled and virus copy number/ml plasma was calculated by real-time RT-PCR. Cutoff for the assay was 10 copies/ml. Boxed area represents treatment period (weeks 2-30 post infection). Open symbols represent animals that received the *gag/tat*/multi-epitope vaccinations and closed symbols represent animals that received the *gag/tat* vaccinations.

*Evolution of PMPA resistance.* Studies have demonstrated that the infected cell population in peripheral blood does not always supply the virus circulating in plasma (53). We demonstrated that the reverse transcriptase mutation, K65R, that confers a 5-fold resistance to PMPA (298) became fixed in the plasma of infected animals that responded poorly to PMPA alone but was present in animals the responded to ART (Chapter 4). To determine if this mutation was present in the integrated provirus from PBMC, thus potentially supplying the plasma virus, we serially monitored this region in untreated, therapeutically immunized, and animals vaccinated pre and post inoculation.

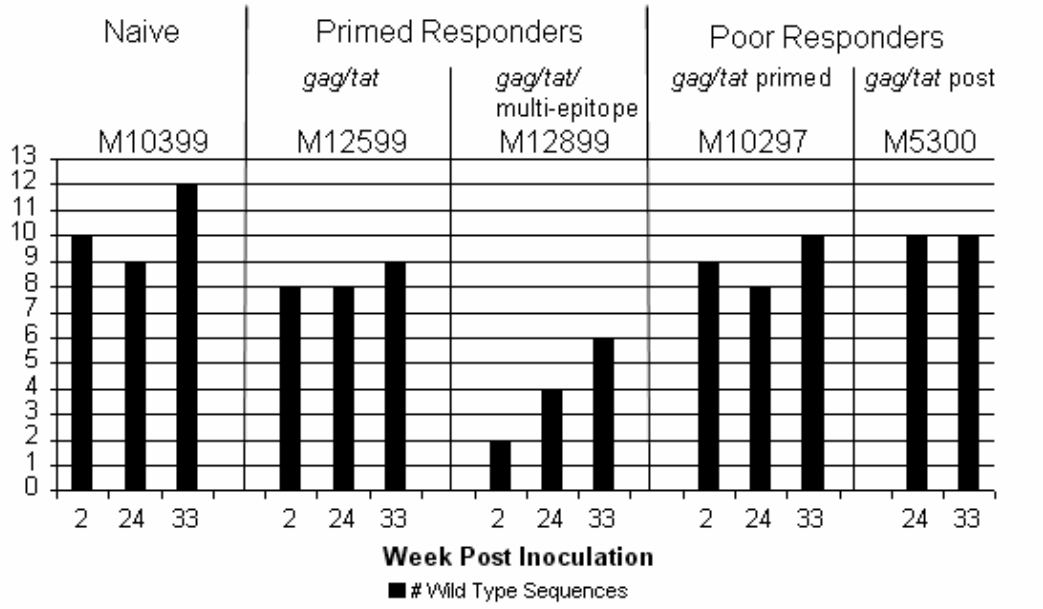
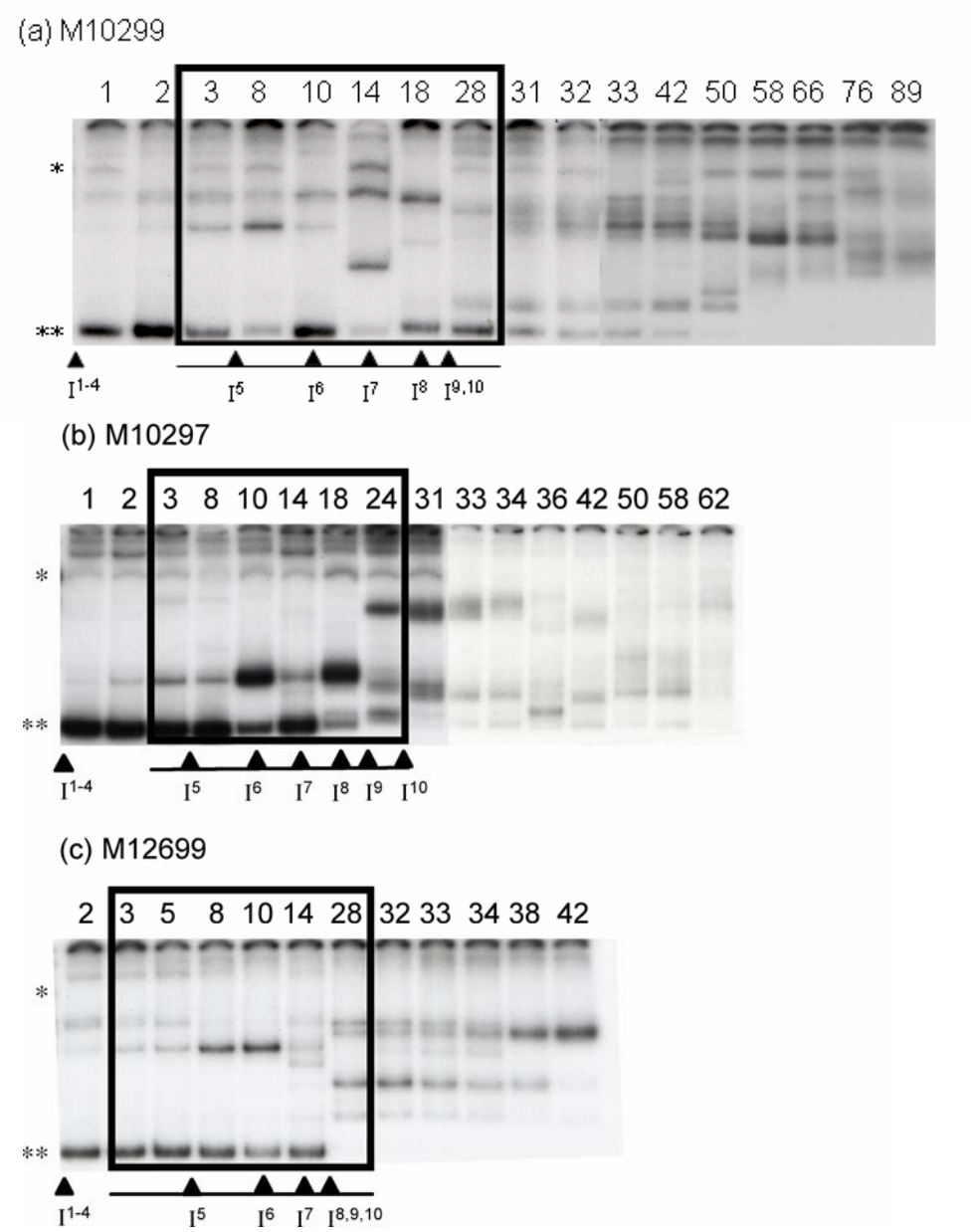



Figure 6.3. Number of clones expressing the wild type (drug sensitive) reverse transcriptase (RT) from proviral PBMC DNA. A portion of the RT gene was amplified from integrated proviral DNA from PBMC at the indicated times post inoculation and sequenced. Black bars represent wild type RT.

We chose to analyze this region before treatment, late during treatment, and three weeks after treatment withdrawal to include the detection of possible reversion mutants. As anticipated, we were unable to detect this mutation from any animal before treatment initiation, or from any time point in the untreated animal (Figure 6.3). Furthermore, we failed to detect this resistance mutation in the PBMC of any animal regardless of which vaccine they received, or response to therapy. This result was surprising because vaccinated poor responders maintained high virus loads in the face of therapy, and our previous findings that the majority of the expressed virus in poor responders given PMPA alone had the resistance mutation. This was in contrast to responders given PMPA alone in which the majority of the circulating plasma virus was drug sensitive and indicates the PBMC may not have been supplying the plasma virus in these animals.

*Viral genetic evolution in vivo*. We performed heteroduplex tacking analysis (HTA) on plasma cDNA sampled longitudinally after infection to determine the effects of the pre post-infection vaccine regimen on virus evolution. At acute infection, HTA revealed the expression of at least one of the major genotypes present in the inoculum, in addition to others, in the vaccine primed animals (Figures 6.3 and 6.4). These results mirrored that seen in animals given drug treatment alone or therapeutic immunizations (Chapters 4 and 5), suggesting that vaccine priming did not impose a genotypic selection bias.

**Poor responders**. The genotypes expressed at acute infection in vaccine primed animals that responded poorly to treatment were maintained approximately 10 weeks pi (Figure 6.3). Throughout the remainder of the treatment and study period, shifts in the dominance of the expressed genotypes occurred, or previously undetected genotypes

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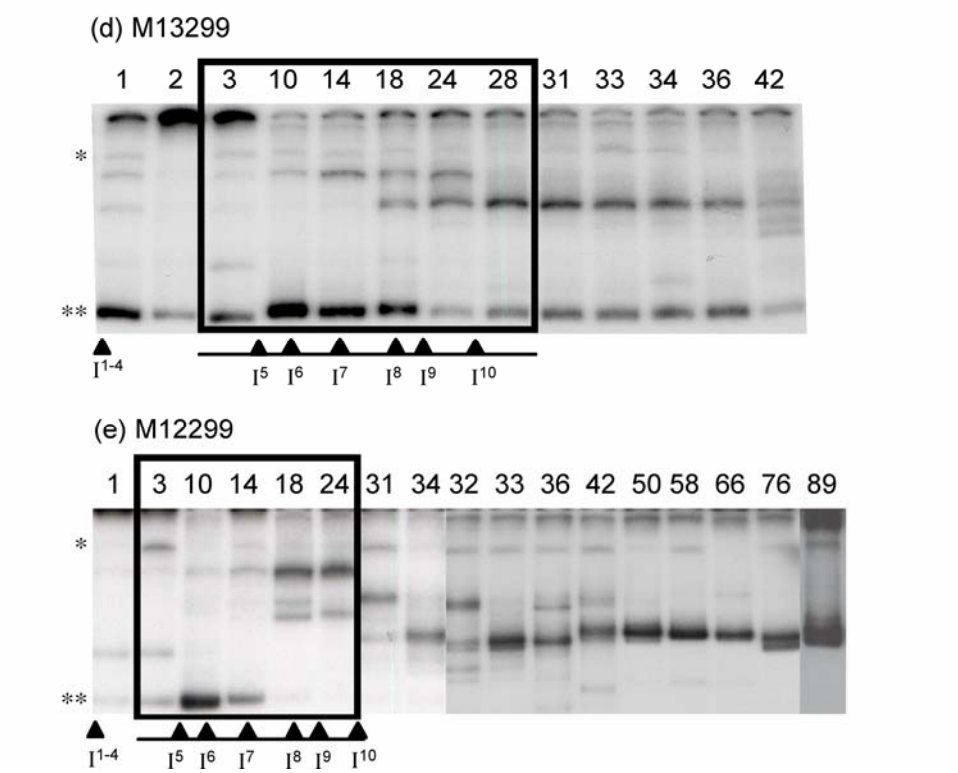
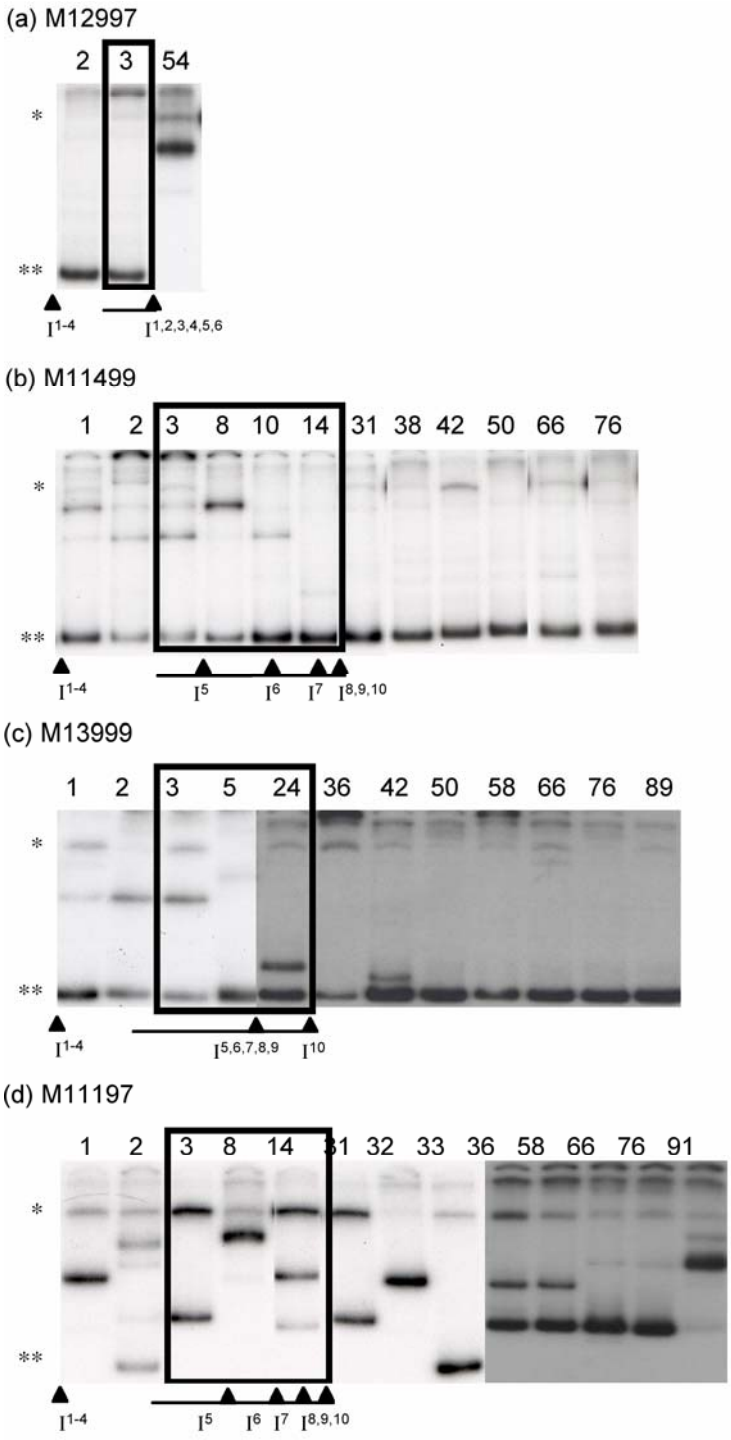



Figure 6.4. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated and immunized pre and post inoculation poor drug responder macaques. Numbers along the bottom indicate week post inoculation. Daily PMPA treatment was initiated 2 weeks pi and continued for 28 weeks. Boxed area represents samples collected during therapy. The last week is the necropsy sample. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. (a) M10299 (b) M10297, (c) M12699, (d) M13299, (e) M12299.were expressed. These results mirrored that seen in poor responders given therapeutic immunizations (Chapter 5, Figure 5.3) or PMPA alone (Chapter 4, Figure 4.5).

**Responders**. During the first eight weeks of therapy (up to week 10 pi), animals that responded to PMPA and received vaccine priming generally maintained expression of genotypes expressed at acute infection (Figure 6.4). During the remainder of therapy, we were unable to amplify viral cDNA from the majority of the responder animals, and from one animal due to a virus burden below our detection limit (M12997), after treatment withdrawal. Six of 11 vaccine animals however, maintained expression of one major genotype until the study was terminated (Figure 6.4, b, c, e, i, and k). The viral quasispecies expressed in the other four animals was more complex (d, f, g, and j). These results also mirrored that seen in responder animals given therapeutic immunizations (Chapter 5, Figure 5.4), or PMPA alone (Chapter 4, Figure 4.6).

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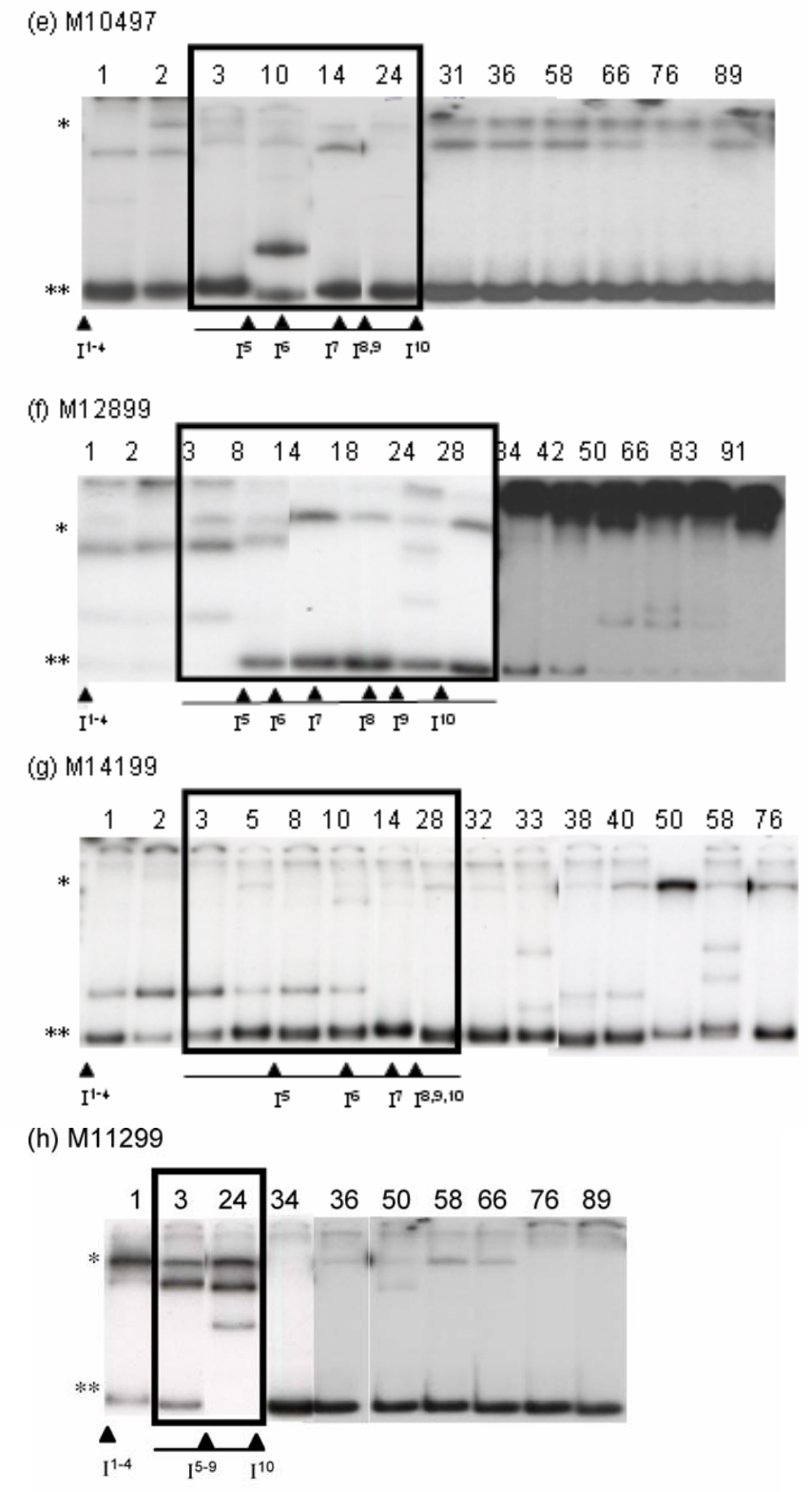


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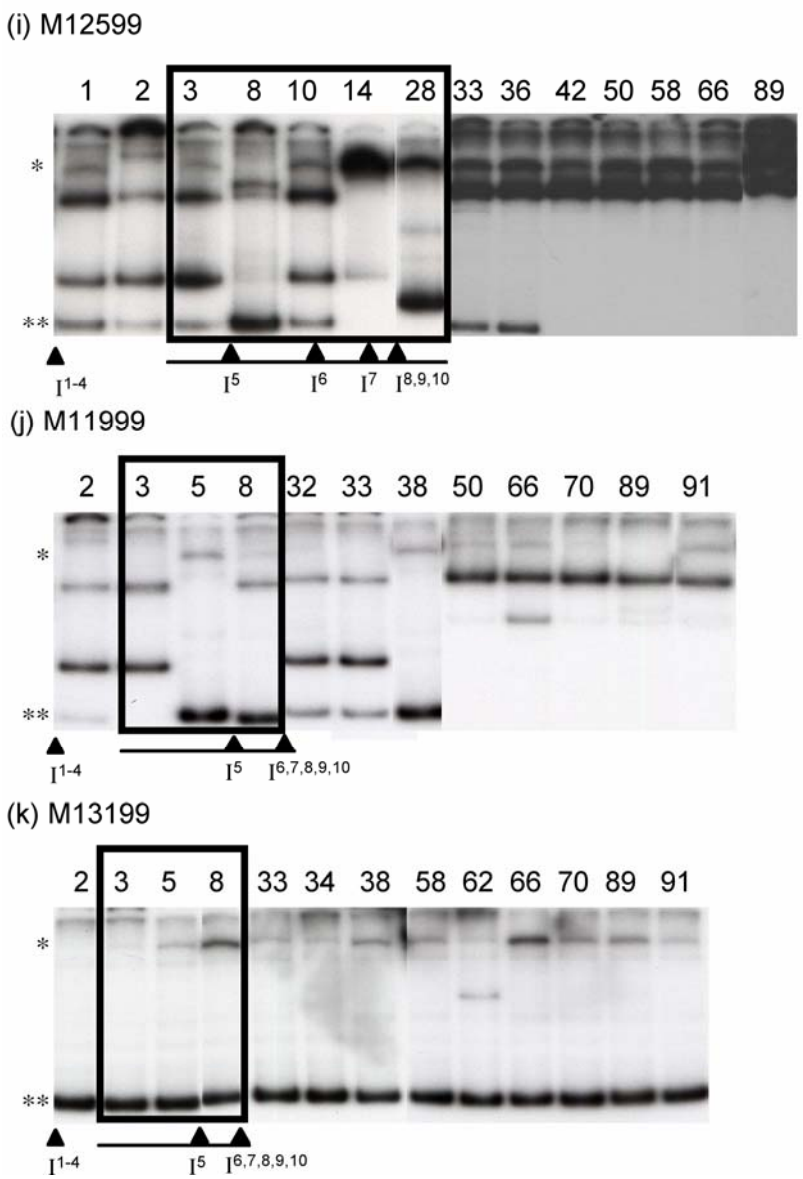


Figure 6.5. HTA of plasma virus cDNA derived V1–V2 envelope region sequences from SIV/DeltaB670-infected, PMPA-treated and immunized pre and post inoculation drug responder macaques. Numbers along the bottom indicate week post inoculation. Daily PMPA treatment was initiated 2 weeks pi and continued for 28 weeks. Boxed area represents samples collected during therapy. The last week is the necropsy sample or with a plasma virus burden above our detection limit. The asterisk and double asterisk indicate single strand probe and homoduplex positions respectively. (a) M12997, (b) M11499, (c) M13999, (d) M11197, (e) M10497, (f) M12899, (g) M14199, (h) M11299, (i) M12599, (j) M11999, (k) M13199.

***Discussion***.

We compared virus evolution between antiretroviral drug treated animals given DNA vaccinations both pre and post inoculation to that seen in animals either vaccinated post infection only (Chapter 5) or drug treated alone (Chapter 4), using a heteroduplex tracking analysis (HTA) of the V1-V2 region of the envelope gene. The vaccine-primed animals displayed the same PMPA responder/poor responder phenotypes seen in the vaccinated and/or drug treated animals. The same DNA vaccine preparation (*gag/tat*/multi-epitope) proved to be the most efficacious, but again worked best in responders. These animals maintained CD4+ T cell counts while the poor responders did not. Vaccine priming induced virus specific T cell responses that were detectable prior to inoculation, and repeated administration during therapy post infection boosted these responses. There was no difference in the acute plasma virus burden among the different groups of animals however. We were also unable to detect a difference in virus evolution between the responder animals given therapeutic immunizations and those that were vaccine primed prior to infection. Again, responder animals, whether immunized or not, expressed a more restricted quasispecies after treatment withdrawal when compared to poor responders.

The importance of the virus specific T cell response to controlling both HIV and SIV virus replication has been demonstrated in a number of studies (35, 142, 158, 193, 256, 307). Thus, we anticipated that vaccine priming might limit virus evolution in vaccinated animals because these animals maintained control of virus replication after treatment withdrawal and this was likely due to T cell control. Vaccine primed responses were unable to induce a change in the genotypes expressed during acute infection,

however. There was also no difference in the quasispecies complexity after treatment withdrawal in primed animals versus those that were treated post infection only.

We had also seen that in animals given drug treatment alone, the virus present in plasma acquired and retained the drug resistance mutation (K65R) throughout the period of analysis in the poor responders (minimum of 30 weeks post drug removal, Chapter 4). To determine whether immunization affected the emergence of drug resistance, we looked for the K65R mutation in PBMC DNA from drug treated vaccinated animals. PBMC DNA was employed in this analysis because our supply of plasma RNA was limited. We reasoned that proviral DNA in PBMC would be representative of the virion RNA, however, because we had shown in another study minimal differences between the two when the V1-V2 region was analyzed by HTA (unpublished results). Surprisingly, despite the rapid appearance of drug resistance mutations in plasma observed in the control animals, only the drug sensitive genotype was identified in PBMC DNA of the vaccine primed group, regardless of their responder phenotype. These results are supported by another study that found a similar discordance in the drug resistant genotype when plasma RNA and PBMC DNA were compared (184). Three of 3 SIV infected macaques examined in the study, which had been on drug therapy a maximum of 20 weeks, had detectable drug resistant genotypes in PBMC provirus whereas all plasma virion genotypes examined were drug sensitive. Together with our own results, these data suggest PBMC may not have supplied the virus circulating in plasma. These results have prompted a more thorough evaluation of lymphoid tissues, PBMC, and plasma sampled longitudinally in a separate study that is underway.

In summary, evaluation of the virologic response to vaccination with a DNA vaccine that induced potent T cell responses failed to identify global genotypic changes associated with host immunity. A number of factors could have contributed to our inability to detect these changes, however, including the vaccine's inability to induce global changes in the virus population, monitoring an uninformative region of the genome (V1-V2), or maintenance of a viral copy number in tissues too low for analysis (Chapter 5 Discussion).

# Chapter 7. Virus Copy Number and Genetic Diversity found in Gut Associated and Peripheral Lymphoid Tissue from SIV Infected Macaques given Antiretroviral Therapies and DNA Vaccines.

***Introduction.***

The resting CD4+ T cell population is known to harbor replication competent proviral DNA in infected individuals (33). Withdrawal of highly active antiretroviral therapy frequently heralds a rebound in the plasma virus burden. Analysis of the rebounding virus has revealed that this resting CD4+ T cell population is not always the source (53). These results suggest the presence of an as yet unknown viral reservoir for persistent virus. During acute infection, the CD4+ T cells in the gut associated lymphoid tissue (GALT) have been shown to be the site of infection and depletion in both HIV infected humans and SIV infected animals (38, 196, 271, 297, 302). The initiation of antiretroviral therapy (ART) early after infection has proven to aid in the reconstitution of the CD4+ T cell population in the GALT in SIV infected macaques (108) and in some HIV infected humans on ART (117). This suggests that the GALT may be one reservoir for virally infected cells in patients receiving ART. An understanding of the effects of ART on the virus found in the GALT could aid in the development of interventions targeted to specific sites or viral variants.

To address this issue we quantitated the virus copy number and analyzed the virus population found in the GALT (mesenteric lymph nodes) and peripheral tissues (axillary and inguinal lymph nodes, and plasma) from SIV infected macaques given ART and DNA vaccinations (Chapter 6). Tissue was collected at the studies conclusion or at necropsy from ART responder and nonresponder animals that survived less than a year after therapy was discontinued (Chapters 4, 5, and 6). To determine the relationship between the plasma virus burden on the virus expressed in the GALT and peripheral

tissues we quantified virus copy number by real time RT-PCR and performed heteroduplex tracking analysis (HTA) on viral cDNA isolated from lymphoid tissues.

***Materials and methods***.

**Animal inoculations, drug treatment, DNA immunizations, and plasma virus load**. Animal inoculations, drug treatment, DNA vaccine preparation, schedule, and quantitation of plasma virus loads were as described (Chapter 6).

**Tissue collection and RNA isolation**. Mesenteric lymph nodes, axillary lymph nodes, inguinal lymph nodes, and jejunal tissues were collected at necropsy due to AIDS or at scheduled sacrifice approximately 2 years post inoculation (see Clinical Tables, Chapter 4, 5, 6). PBMC were collected only from animals at scheduled sacrifice. Tissue was snap frozen in OCT and stored at –80°C until use. 100 mg tissue sections were placed in 1 ml Trizol reagent and total RNA was extracted per manufacturers instructions (Invitrogen, Carlsbad, CA). RNA samples were stored at –80 °C before use. To determine if the samples contained geneomic DNA contamination, the V1-V2 region of the envelope gene was amplified from 2 μl of total RNA by PCR using the previously described primers and cycling parameters (287). Ten μl of the product was run on an agarose gel and if the sample yielded a positive result the RNA was treated with DNase per manufacturers instructions (Ambion, DNA free RNA, Austin TX). The PCR was repeated and only samples free of DNA contamination were analyzed further. All reagents were prepared with DEPC water.

**Tissue virus copy number**. The virus copy number from 500 ng of RNA was quantitated by real time RT-PCR as described (Chapter 6). Expression of β-glucuronidase (GUS) was quantitated from 500 ng of RNA as an control of expression. The primers and probe were as described (109 J Molecular Diagnostics 2:84). Data are expressed as the ratio of individual tissue viral copies to individual β-GUS/ total β-GUS.

**Heteroduplex tracking analysis (HTA)**. HTA was performed as described (Chapter 4) using only DNA free RNA. Samples were run in duplicate to confirm the results were identical. Only identical duplicate samples were evaluated further.

***Results***

*Study design.* To determine the amount of virus in lymphoid tissue, and whether the GALT served as a reservoir for unique viral variants, total RNA was extracted from mesenteric, axillary, and inguinal lymph nodes and jejunal tissue at necropsy from all of the animals in the study (Chapters 4, 5, and 6). RNA samples found to contain contaminating DNA were not included in the analysis. The virus copy number/500 ng total RNA was quantitated by real time RT-PCR. The V1-V2 region of the envelope gene was also amplified from cDNA and analyzed by HTA to determine the genotypic expression patterns.

*Tissue Virus loads*. We stratified the normalized tissue virus copy number according to the plasma virus copy number at necropsy and compared the copy numbers between the GALT (mesenteric lymph node, jejunal lamina propria) and peripheral lymph nodes (axillary and inguinal) within a range of plasma virus burdens ($0 < 10^4$ and $> 10^4$ viral copy numbers/ml plasma) (Figure 7.1). This broke the samples into tissue from responders ($0 < 10^4$ viral copy numbers/ml plasma), and poor responders ($> 10^4$ viral copy numbers/ml plasma), excluding five animals with the highest virus burdens at necropsy (monkeys M9699, M10999, M5200, M4900, and M11999) that were originally characterized as responders during therapy (Chapters 4, 5). These five animals had all demonstrated viral rebound after treatment withdrawal. A comparison of the virus copy numbers between tissues within the lower range of plasma virus burden, revealed that jejunal tissue had significantly less virus than the other tissues (Table 7.4. $P < 0.005$, Mann-Whitney U test, 2 tailed). These results could indicate a continuous loss of the infected CD4+ T cell population throughout the study, the majority of which continued

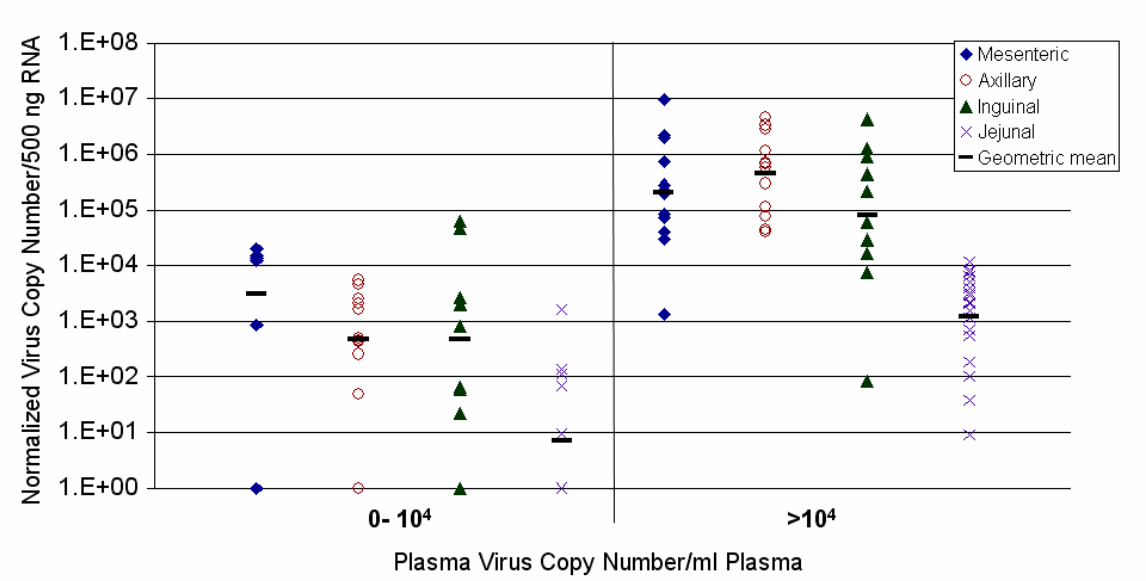


Figure 7.1. Tissue virus copy number/500 ng total RNA normalized to the expression of β-GUS. Total RNA was isolated from the indicated bulk tissues collected at necropsy or the studies conclusion, approximately 2 years post inoculation. Results are stratified according to the plasma virus copy number/ml plasma.

Table 7.4. Statistical analysis of the tissue virus burdens.

| Plasma Virus Load[1] | Tissues Compared | | | | |
|---|---|---|---|---|---|
| | | Mesenteric | Axillary | Inguinal | Jejunal |
| 0 Š $10^4$ | Mesenteric | | 0.016[2] | 0.321 | 0.001 |
| | Axillary | | | 1 | 0.002 |
| | Inguinal | | | | 0.009 |
| < $10^4$ | Mesenteric | | 0.189 | 0.248 | 0.000 |
| | Axillary | | | 0.180 | 0.000 |
| | Inguinal | | | | 0.000 |
| 0 Š $10^4$ to < $10^4$ | Mesenteric | 0.000 | | | |
| | Axillary | | 0.000 | | |
| | Inguinal | | | 0.000 | |
| | Jejunal | | | | 0.000 |

[1]Virus copy number/ml plasma at necropsy.
[2]P value, Mann-Whitney U Test, 2 Tailed

without ART in the responders. The difference between the mesenteric lymph nodes and the axillary lymph nodes was also significant (P 0.028, Mann-Whitney U test, 2 tailed). However, no difference in virus copy number was seen when comparing either the mesenteric or axillary lymph node (LN) to the inguinal LN tissues (P = 0. 321 and 1 respectively, Mann-Whitney U test, 2 tailed).

We compared the tissue virus burdens between the high range of plasma virus burdens and found there was no difference between the mesenteric, axillary and inguinal LNs (P > 0.180, Mann-Whitney U test, 2 tailed). Additionally, we also observed a significant difference between the virus loads in any of these tissues and the jejunum (P < 0.000, Mann-Whitney U test, 2 tailed). The difference in the mesenteric, axillary and inguinal LN copy numbers between the low and high plasma virus loads was always significant (P >0.000, Mann-Whitney U test, 2 tailed). Together these results suggest a higher plasma virus burden results in a higher lymphoid tissue virus load.

*Tissue HTA.* The infection of the GALT during acute infection could enable this as a reservoir for unique and potentially ancestral virus that may not remain transcriptionally active as disease progresses. To determine if this was the case, we performed heteroduplex tracking analysis (HTA) on cDNA prepared from both GALT and peripheral tissue RNA collected at necropsy from SIV infected animals. We isolated total RNA from the lymphoid tissues, and PBMC from the 44 animals in the study. Only samples that were free of DNA contamination, with virus copy numbers above $10^3$, that resulted in identical HTA patterns when analyzed in duplicate, and in which we had a complete set of tissue samples were included in the analysis (Figure 7.2). Our requirement for identical HTA duplicates resulted in the omission of monkey, M13899

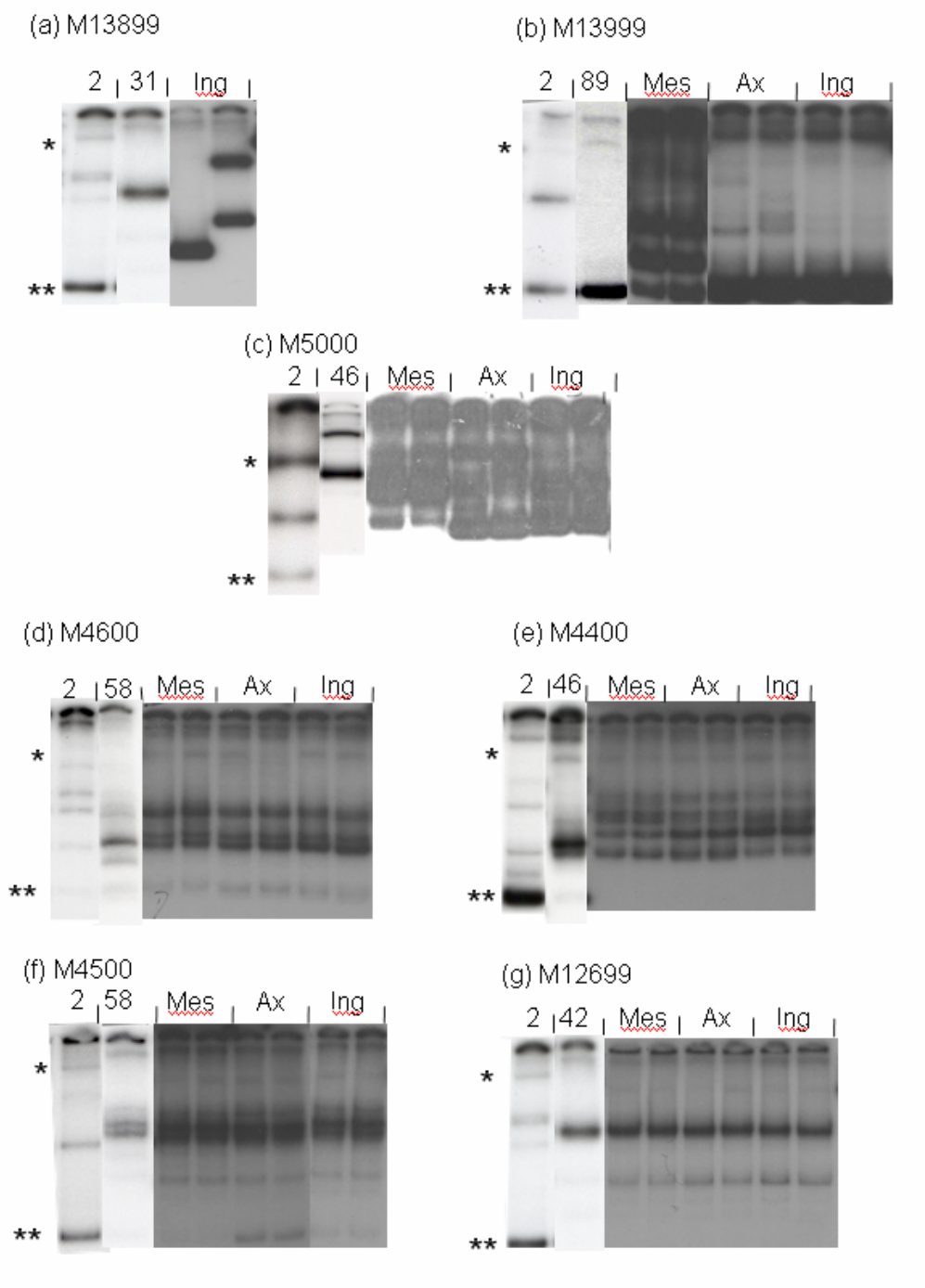


Figure 7.2. HTA of viral cDNA from plasma and tissue from drug responder animals. cDNA was prepared from plasma virus at the weeks post inoculation indicated at the top of the figures. cDNA was prepared from total RNA isolated at necropsy from the indicated tissue. All samples were prepared independently and run together to confirm sampling represented the entire virus population. Mes, mesenteric lymph node, Ax, axillary lymph node, Ing, inguinal lymph node. Asterisk and double asterisk indicate the single strand probe and homoduplex positions respectively.

(Figure 7.2, included as an example) and the axillary LN from M13999. Additionally, this precluded our analysis of the jejunal tissues and PBMC.

HTA revealed that the majority of genotypes found in the GALT were also found in the peripheral tissues (Figure 7.2). HTA also revealed that the axillary and inguinal LN from one animal contained genotypes not found in the GALT (monkey M4500). However, two animals, M13999 and M5000 harbored genotypes unique to the mesenteric LN.

We also compared the tissue and plasma HTA to determine if genotypes found in the tissues were also expressed in the plasma at necropsy. This analysis showed that the dominant genotype expressed in plasma was also found in the tissues from four of five animals (M5000 was not included because the plasma HTA from necropsy was not available due to a plasma virus burden below our detection limit). A minor variant was also found in both plasma and the tissues from the fifth animal (M4400). These results demonstrate that at necropsy there is a wide distribution of genotypes. We also compared the genotypes expressed at acute infection (week 2 pi) and tissue samples to determine if the tissues expressed genotypes that could represent archival genotypes. We found that one animal that survived until the studies conclusion (M13999) and two that succumbed to AIDS (monkeys M4600 and M4500) had genotypes similar to variants expressed during acute infection.

***Discussion***.

We quantitated the virus copy number/500 ng total RNA from lymphoid tissues obtained at necropsy from SIV infected animals given ART with and without therapeutic DNA immunizations. These samples were collected over a year after the drug and immunizations had been discontinued. We also characterized the genotypes found in these tissues by HTA and compared them to genotypes expressed in plasma.

HIV/SIV rapidly disseminates among lymphoid tissue compartment early after infection (14, 98, 275). In animals that control virus replication, we anticipated that fewer cells throughout the body would be infected. To analyze this, we compared the cell-associated virus loads in tissues from animals with high ($>10^4$ copies/ml plasma) and low ($>10^4$ copies/ml plasma) virus copy number. The cell-associated RNA virus copy numbers in axiallary, inguinal, and mesenteric lymph nodes were consistent with the amount of virus in plasma, with the control of virus replication resulting in lower virus copy numbers in the tissues, and higher plasma virus burdens having higher tissue virus RNA numbers. These results are supported by earlier studies that made a similar observation in untreated HIV infected individuals (98) and SIV infected macaques (161), and infected animals given ART one weeks post infection (273).

Dissemination of the virus throughout the body is accompanied by early depletion of the activated CD4+ T cell population in the lamina propria of the gut following both HIV and SIV infection (38, 196, 302, 303). Studies have also demonstrated there is only a limited repopulation of this cell population even then ART is initiated within one week of infection (176). Thus, it was not surprising that the viral RNA copy numbers found in our animals were lower in jejunal tissues when compared to that found in the other

tissues. These results suggest that although ART was initiated early after infection, its withdrawal and the subsequent disease progression (however limited) resulted in an inability to repopulate the CD4+ T cells in these animals even a year after drug was discontinued and virological control was maintained.

The robust induction of virus specific T cell responses are associated with control of virus replication (35, 142, 158, 193, 256, 307). Higher plasma virus copy numbers associated with a failure to control repliction would logically be associated with expression of a complex quasispecies that would be disseminated among all lymphoid compartments. Control of virus in the peripheral circulation, on the other hand, is nevertheless not absolute. We therefore questioned whether the origin(s) of the persistent virus found in the circulation could be confined to specific organs. Some studies have identified distinct viral populations in different tissues or organs (19, 24, 77, 240). This study represents the first report of the dissemination patterns for SIV/DeltaB670 genotypes into organs or tissues other than the mucosa (287). Analysis of the genotypes found in the lymphoid tissues demonstrated there was wide dissemination of genotypes with advanced disease, with the majority of the genotypes found in all of the analyzed tissues. These results indicate there was no site specific selection pressures on the different DeltaB670 genotypes during active disease progression and are supported by an earlier mucosal study from our laboratory that did not find a transmission bias of genotypes across the mucosal barrier (287). Studies performed by others have made similar observations, however these reports analyzed different organ systems than those reported here (41, 156).

We hypothesized that the GALT could serve as a reservoir of latent virus in animals that responded well to ART. If so, then unique viral genotype(s) would be expressed in some, but not all tissues in the GALT. Because we limited our analysis to only those samples that were free of DNA contamination, had virus copy numbers above $10^3$, identical HTA patterns when analyzed in duplicate, and a complete set of tissues for comparison purposes, the number of samples evaluated was small. However, in 2/2 animals that maintained control of the infection we detected unique genotypes expressed in the mesenteric lymph nodes. This was in contrast to four animals that were unable to control virus replication wherein the majority of tissues expressed the same animal specific genotypes. These data suggest that the GALT can selectively be infected and persistently express unique genotypes that perhaps may serve as the inoculum as the disease progresses.

These studies are important because they lend insight into the viral replication dynamics in animals controlling infection versus those that succumbed to AIDS. Although these studies were limited by our ability to analyze virus evolution at only one time point, they represent a basis for further analysis of longitudinally sampled tissues throughout infection.

# Chapter 8. Comprehensive Discussion

In 2004 alone approximately 3 million people died from acquired immunodeficiency syndrome as a direct consequence of infection by the human immunodeficiency virus (294). An estimated 40 million people are currently infected with HIV (295) and will die if life saving interventions are not developed. The rapid adaptability of the virus to new environments has thus far made eradication impossible.

Viral evolution is driven by both viral and host factors (26, 31, 36, 59, 67, 100, 113, 128, 141, 155, 170, 173, 188, 204, 244, 245, 262, 265, 280, 310, 311, 326, 330). HIV-1 is believed to have arisen from three separate cross-species transmissions of chimpanzee SIV (SIVcpz) into humans while HIV-2 arose from a similar zoonotic transmission of sooty mangabey viruses (SIVsm) (72, 169, 263, 264). SIVcpz and SIVsm are not known to cause disease within their respective natural hosts (118) while few infected humans are known to remain free of the devastating effects of infection (21, 39, 46, 246). Understanding the interplay between host and virus and the significance that genetic adaptation plays in this process is critical to the development of therapeutics effective in disease control. Therapeutic drug treatments have become available to the developed world and have provided an extension of the disease free period to those who respond optimally (83, 91, 233). Numerous vaccine trials are also underway in an attempt to either prevent infection, or to provide a therapeutic benefit to those already infected (information on prophylactic and therapeutic vaccine trials is available at http://www.aidsinfo.nih.gov/Vaccines/Default.aspx). Thus far, the protective effects of therapy have been limited by the mutation of viral genes that can reduce the susceptibility of the virus to either drug treatment (20, 64) or to the induced immune response (9, 228).

The focus of this body of work was to study the effects of antiretroviral therapy (ART) with and without DNA vaccination, on virus evolution *in vivo* to determine if there is a relationship between genetic diversity and clinical outcome. The overarching hypothesis of this work was that a strong virus specific immune response induced either by a controlled infection or by immunization with a potent T cell vaccine, would limit virus replication to the point that evolution of the viral quasispecies would be slowed or stopped. This event would then lead to a more stationary target for the immune response and result in a better clinical outcome. We additionally hypothesized that the gut associated lymphoid tissue (GALT) would serve as a reservoir for virus production, and perhaps be revealed by expression of genotypes unique to the site, as a result of maintenance of archival virus. Animals that were infected and untreated, given short-term ART alone, or treated with ART in combination with DNA vaccines, were used for analysis (102).

The vaccines were designed to induce/enhance the virus specific T cell response through the inclusion of the entire coding sequences of the SIV-Fr Gag and Tat proteins. These were administered either alone or in combination with a multi-epitope cocktail encoding known Mama-A*01 epitopes (10). Vaccinations were delivered either post infection only or pre and post infection. Post infection immunizations were administered during treatment with the antiretroviral drug PMPA which was initiated two weeks post infection and was continued daily for 28 weeks. Treated animals responded differently to PMPA and displayed either a drug responder or poor responder phenotype. Drug responders maintained a plasma virus burden at or below $10^4$ virus copies/ml plasma during ART whereas nonresponders had a virus burden of greater than $10^4$ virus

copies/ml plasma. The goal of the immunotherapy was to augment the reduction in the virus burden associated with drug therapy and prevent viral rebound once drug was discontinued. Thus, efficacy was defined as maintenance of the CD4+ cell population for one year coupled with maintenance of a plasma virus load below $10^4$ for six months after ART withdrawal. Efficacy was observed only in responder animals that received the *gag/tat*/multi-epitope immunizations. This effect was evident in both post infection only and pre and post infection immunization groups and was associated with higher and broader virus specific T cell response.

The viral quasispecies was monitored longitudinally in plasma and in lymphoid tissues at necropsy in all animals by heteroduplex tracking analysis (HTA). Analysis of virus expressed in plasma was performed because virus in the circulation is representative of the genotypes associated with disease progression. Analysis of the genotypes expressed in the tissues was performed because this knowledge should provide important information regarding organ reservoirs of virus during chronic infection. Together these studies should aid in the design of future antiviral therapies by identifying genotypes and/or organs for targeted interventions.

We first optimized HTA for use with the challenge isolate, DeltaB670. One of the two dominant genotypes in the inoculum (clone 3) was subsequently shown to be at a replication disadvantage in primary rhesus PBMC when compared to the second genotype (clone 12). Conversely, clone 12 was selected against by in vitro propagation of the stock in human T cell lines. Since this result was demonstrated *in vitro* without immune mediated selection pressure, it supports the conclusion that lost of clone 3 *in vivo* would be due to poor replication fitness and not immune clearance. Analysis of plasma

cDNA demonstrated that expression of clone 3 was lost in the majority of animals (10/16) that responded poorly to drug treatment. Surprisingly, expression of this clone was better preserved in animals that responded to ART, whether they received vaccinations or not (11/19; we were unable to identify the expressed genotypes in 5 animals due to expression levels below our limit of detection). These results suggest that the reduction in virus burden associated with a good response allowed maintenance of the poorly fit (attenuated) genotype and prevented (more virulent) replication/infection of the monkey adapted viruses thereby aiding immunotherapy at the molecular level. A fitness advantage of particular genotypes has been demonstrated previously *in vitro* (306) and *in vivo* (130, 162). A direct correlation between viral evolution and disease progression has not yet been identified. Even though the use of different co-receptors for entry was shown to allow replication in different cell types and resulted in depletion of the CD4+ T cell population (262, 327) a progression to AIDS is still seen in individuals infected with HIV isolates that do not demonstrate a co-receptor switch. Attenuated disease progression has also been demonstrated in some HIV+ individuals infected with nef-deleted viral isolates (151, 165, 200). This deletion has not always been shown to be associated with long-term nonprogressor status, however (134, 135). Taken together, these results suggest a dominant role for host factors in disease progression.

Host factors that influence disease progression have been identified in HIV infected individuals (195, 199, 227, 247) and SIV infected macaques (175, 236). A differential response to PMPA in our study was also evident by five weeks post inoculation, suggesting host factors likely played a dominant role in drug responsiveness. This result prompted us to look at host factors present before the initiation of ART. We

compared the acute virus burden and the magnitude of the T cell response in animals given drug treatment alone and found there was no difference in these parameters. However, although the numbers of animals were limited, a comparison of the number of viral variants expressed in responder and poor responder animals in the PMPA treated control group during acute infection neared statistical significance (P = 0.057, Mann-Whitney U test, 2 tailed), with the responder animals expressing fewer viral genotypes than the poor responders. We were unable to compare these parameters between the vaccinated animals because response to ART was determined during the period of PMPA therapy when the animals were also vaccinated and the vaccines could have additionally influenced drug responsiveness. Previous studies in our laboratory however, have demonstrated *in vitro* that primary cell cultures from some animals have faster and higher replication kinetics than that seen in cells from other animals (261). These results were mirrored *in vivo* and resulted in higher plasma virus burdens and a more rapid disease progression. It was subsequently determined that this result was due to post entry inhibition of the early stage of reverse transcription (123). Although the remainder of the virus life cycle was apparently unaffected, his block resulted in fewer cells becoming infected in subsequent rounds. During reverse transcription, partial uncoating of the viral core renders the virus susceptible to inhibitory host factors (265, 280) that could influence the subsequent disease course. We can envision that host factors working in concert could influence not only the clinical disease course, but also the response to ART by differentially suppressing the replication of monkey adapted genotypes Confirmation of this hypothesis must await further analysis of a larger cohort of animals.

We also demonstrated by HTA that there was less evidence of viral evolution in animals that responded to PMPA, again, whether they received vaccinations or not. These results suggest that none of the vaccines were able to affect particular genotypes even though they were shown to help in suppressing the virus population over that seen by drug treatment alone. Virus specific T cell immune responses were enhanced, both in magnitude and breadth, by vaccination when compared to that seen with infection alone. A protective immune response has not yet been identified for HIV. In some studies the neutralizing antibody response has been associated with protective effects (192, 223, 243) while in other studies the CTL response has been correlated with protection (124, 215, 312). Alternatively, selection of escape mutants in the immunodominant Gag epitope CM9 occurs only late in infection in Mamu-A*01 animals. This finding could reflect an ability to replicate under immune mediated pressure (210). Together these results indicate that numerous factors could be combining to contribute to both disease progression and response to ART, with both antibody and T cell responses likely playing dominant roles.

A positive effect on clinical outcome with PMPA treatment has been shown in both HIV and SIV infection even when infection is dominated by drug-resistant virus (22, 180, 258, 299, 300). PMPA has been shown to have immunomodulatory effects in murine models *in vitro* and *in vivo*. The effects include enhancing natural killer cell activity, inducing secretion of both cytokines and chemokines, and enhancing the *in vivo* resistance to viruses that are not sensitive to the drug *in vitro* (45, 74, 217, 305, 331). Although these effects have not yet been demonstrated in the macaque model, *in vitro* studies have demonstrated PMPA primes rhesus PBMC to upregulate IL-12 secretion after exposure to bacterial antigens (300). IL-12 has a diverse role in strengthening

innate immunity, including reducing apoptosis (241, 288). An enhanced T cell response and a reduced level of CD8+ T cell apoptosis has been demonstrated in macaques given four weeks of PMPA initiated one week after infection that was not seen in untreated animals (273). Together these effects could have rendered a replication advantage of the clone 3 genotype, possibly through the combined effects of yet unidentified viral and host factors in the responder animals, even though they were unable to eliminate this genotype.

The GALT contained virus copy numbers similar to that observed in peripheral tissues in animals with high virus loads in the plasma. However, mesenteric lymph nodes had higher levels of virus and expressed unique genotype(s) in two animals that maintained control of the infection. This suggests that this organ may serve as a reservoir of persistent expression during clinical latency. A more definitive study in this regard will be to serially sample these organs over time during ART.

A high degree of conservation existed between epitope sequences used in the vaccine and those encoded by the challenge virus. Thus, effective immunization should limit the replication of genotypes expressing these epitopes. Indeed, an earlier study using the same vaccine constructs and DeltaB670 as the challenge virus resulted in complete protection in 4/7 animals challenged mucosally. Evolution of the viral quasispecies was not evaluated in this study, however. In the present study we observed no difference in either the plasma virus burden, or the genotypes expressed at acute infection between any of the groups. Together these results suggest that the vaccine was unable to induce global changes in the viral quasispecies.

**Future Directions**.

The analysis of the virus burden and expressed genotypes provide the first snap shot of events later in infection in animals given ART and DNA vaccines. The lack of a demonstrable difference in evolution evident by HTA in the animals that responded to, and received only ART, and the vaccine strategies employed in this study is intriguing. Monitoring additional regions encoded by the vaccine, in tandem with the V1-V2 envelope sequences, may demonstrate if changes in specific epitope(s) have occurred without loss of the associated V1-V2 region.

A comprehensive analysis of nonsynonymus changes in T cell epitope sequences combined with the ability of the host to respond immunologically to them over time in infected macaques should answer this question.

The limited evidence that mesenteric lymph nodes may harbor and express distinct viral genotypes during chronic infection also bears further study. Because the study was limited to a cross-sectional analysis of monkeys late in infection that either controlled or succumbed to infection, how virus evolves over time within these tissues remains an open question. Serial sampling of organs throughout infection would provide a clearer picture of events occurring locally, and their association with disease progression. This knowledge could potentially aid in the design of future intervention strategies by identifying epitopes/genotypes for targeted intervention.

In summary, despite the large amount of heterogeneity between the main subgroups of HIV, analysis of the available sequence data demonstrate there are clear consensus sequences that suggest there is a fitness cost to unrestrained genetic variability. Indeed, reversion to a consensus sequence has been suggested upon transmission of an HIV CTL escape mutant into a recipient with a different HLA type (112, 170, 206). Such viral reversion has been demonstrated in the SIV model (100, 154). The fitness cost is readily demonstrated when analyzing reversion of drug resistance mutations in the absence of drug therapy to wild-type sequences in both HIV (104, 119) and SIV infections (216). This indicates that designing a vaccine that can target a broad number of epitopes, preferably those that require multiple mutations for escape, could minimally prolong the disease free period of HIV infected individuals. This will require monitoring virus evolution at an epitope level and possibly the genome level when designing vaccine strategies to 1)-identify epitopes that require more numerous nucleotide substitutions for escape and 2)-to identify attenuation in other regions of the genome that contribute to delayed disease progression.

# Bibliography

1. 1990. Interim proposal for a WHO Staging System for HIV infection and Disease. Wkly Epidemiol Rec **65:**221-4.
2. 1981. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. MMWR Morb Mortal Wkly Rep **30:**305-8.
3. 1991. Structural organization and biological properties of langerhans cells. Schuler, Boca Raton.
4. 2000. Survival after introduction of HAART in people with known duration of HIV-1 infection. The CASCADE Collaboration. Concerted Action on SeroConversion to AIDS and Death in Europe. Lancet **355:**1158-9.
5. **Aasa-Chapman, M. M., A. Hayman, P. Newton, D. Cornforth, I. Williams, P. Borrow, P. Balfe, and A. McKnight.** 2004. Development of the antibody response in acute HIV-1 infection. Aids **18:**371-81.
6. **Ahmed, R., B. D. Jamieson, and D. D. Porter.** 1987. Immune therapy of a persistent and disseminated viral infection. J Virol **61:**3920-9.
7. **aidsinfo**, US Department of Health and Human Services. Vaccines. http://aidsinfo.nih.gov/Vaccines/Default.aspx?MenuItem=Vaccines
8. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo.** 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. Aids **4:**107-12.
9. **Allen, T. M., M. Altfeld, S. C. Geer, E. T. Kalife, C. Moore, M. O'Sullivan K, I. Desouza, M. E. Feeney, R. L. Eldridge, E. L. Maier, D. E. Kaufmann, M. P.**

**Lahaie, L. Reyor, G. Tanzi, M. N. Johnston, C. Brander, R. Draenert, J. K. Rockstroh, H. Jessen, E. S. Rosenberg, S. A. Mallal, and B. D. Walker.** 2005. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. J Virol **79:**13239-49.

10. **Allen, T. M., B. R. Mothe, J. Sidney, P. Jing, J. L. Dzuris, M. E. Liebl, T. U. Vogel, D. H. O'Connor, X. Wang, M. C. Wussow, J. A. Thomson, J. D. Altman, D. I. Watkins, and A. Sette.** 2001. CD8(+) lymphocytes from simian immunodeficiency virus-infected rhesus macaques recognize 14 different epitopes bound by the major histocompatibility complex class I molecule mamu-A*01: implications for vaccine design and testing. J Virol **75:**738-49.

11. **Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins.** 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **407:**386-90.

12. **Allen, T. M., J. Sidney, M. F. del Guercio, R. L. Glickman, G. L. Lensmeyer, D. A. Wiebe, R. DeMars, C. D. Pauza, R. P. Johnson, A. Sette, and D. I. Watkins.** 1998. Characterization of the peptide binding motif of a rhesus MHC class I molecule (Mamu-A*01) that binds an immunodominant CTL epitope from simian immunodeficiency virus. J Immunol **160:**6062-71.

13. **Alter, H. J., J. W. Eichberg, H. Masur, W. C. Saxinger, R. Gallo, A. M. Macher, H. C. Lane, and A. S. Fauci.** 1984. Transmission of HTLV-III infection from human plasma to chimpanzees: an animal model for AIDS. Science **226:**549-52.

14. **Altfeld, M., J. van Lunzen, N. Frahm, X. G. Yu, C. Schneider, R. L. Eldridge, M. E. Feeney, D. Meyer-Olson, H. J. Stellbrink, and B. D. Walker.** 2002. Expansion of pre-existing, lymph node-localized CD8+ T cells during supervised treatment interruptions in chronic HIV-1 infection. J Clin Invest **109:**837-43.

15. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science **292:**69-74.

16. **Amedee, A. M., N. Lacour, J. L. Gierman, L. N. Martin, J. E. Clements, R. Bohm, Jr., R. M. Harrison, and M. Murphey-Corb.** 1995. Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. J Virol **69:**7982-90.

17. **Arion, D., G. Borkow, Z. Gu, M. A. Wainberg, and M. A. Parniak.** 1996. The K65R mutation confers increased DNA polymerase processivity to HIV-1 reverse transcriptase. J Biol Chem **271:**19860-4.

18. **Baba, T. W., Y. S. Jeong, D. Pennick, R. Bronson, M. F. Greene, and R. M. Ruprecht.** 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. Science **267:**1820-5.

19. **Babas, T., J. B. Dewitt, J. L. Mankowski, P. M. Tarwater, J. E. Clements, and M. C. Zink.** 2006. Progressive selection for neurovirulent genotypes in the brain of SIV-infected macaques. Aids **20:**197-205.

20. **Bagnarelli, P., S. Menzo, A. Valenza, S. Paolucci, S. Petroni, G. Scalise, R. Sampaolesi, A. Manzin, P. E. Varaldo, and M. Clementi.** 1995. Quantitative molecular monitoring of human immunodeficiency virus type 1 activity during therapy with specific antiretroviral compounds. J Clin Microbiol **33:**16-23.

21. **Balotta, C., P. Bagnarelli, C. Riva, A. Valenza, S. Antinori, M. C. Colombo, R. Sampaolesi, M. Violin, M. P. de Pasquale, M. Moroni, M. Clementi, and M. Galli.** 1997. Comparable biological and molecular determinants in HIV type 1-infected long-term nonprogressors and recently infected individuals. AIDS Res Hum Retroviruses **13:**337-41.

22. **Barditch-Crovo, P., S. G. Deeks, A. Collier, S. Safrin, D. F. Coakley, M. Miller, B. P. Kearney, R. L. Coleman, P. D. Lamy, J. O. Kahn, I. McGowan, and P. S. Lietman.** 2001. Phase i/ii trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. Antimicrob Agents Chemother **45:**2733-9.

23. **Barlow, K. L., J. Green, and J. P. Clewley.** 2000. Viral genome characterisation by the heteroduplex mobility and heteroduplex tracking assays. Rev Med Virol **10:**321-35.

24. **Barnett, S. W., A. Barboza, C. M. Wilcox, C. E. Forsmark, and J. A. Levy.** 1991. Characterization of human immunodeficiency virus type 1 strains recovered from the bowel of infected individuals. Virology **182:**802-9.

25. **Barouch, D. H., A. Craiu, S. Santra, M. A. Egan, J. E. Schmitz, M. J. Kuroda, T. M. Fu, J. H. Nam, L. S. Wyatt, M. A. Lifton, G. R. Krivulka, C. E. Nickerson, C. I. Lord, B. Moss, M. G. Lewis, V. M. Hirsch, J. W. Shiver, and N. L. Letvin.** 2001. Elicitation of high-frequency cytotoxic T-lymphocyte responses against both dominant and subdominant simian-human immunodeficiency virus epitopes by DNA vaccination of rhesus monkeys. J Virol **75:**2462-7.

26. **Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature **415:**335-9.

27. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-71.

28. **Baskin GB, Martin LN, Rangan SR, Gormus BJ, Murphey-Corb M, Wolf RH, and S. KF.** 1986. Transmissible lymphoma and simian acquired immunodeficiency syndrome in rhesus monkeys. J Natl Cancer Inst **77:**127-39.

29. **Beger, E., B. Deocharan, M. Edelman, B. Erblich, Y. Gu, and C. Putterman.** 2002. A peptide DNA surrogate accelerates autoimmune manifestations and nephritis in lupus-prone mice. J Immunol **168:**3617-26.

30. **Benveniste, R. E., W. R. Morton, E. A. Clark, C. C. Tsai, H. D. Ochs, J. M. Ward, L. Kuller, W. B. Knott, R. W. Hill, M. J. Gale, and et al.** 1988. Inoculation of baboons and macaques with simian immunodeficiency virus/Mne, a primate lentivirus closely related to human immunodeficiency virus type 2. J Virol **62:**2091-101.

31. **Bernardin, F., D. Kong, L. Peddada, L. A. Baxter-Lowe, and E. Delwart.** 2005. Human immunodeficiency virus mutations during the first month of infection are preferentially found in known cytotoxic T-lymphocyte epitopes. J Virol **79:**11523-8.

32. **Birx, D. L., L. D. Loomis-Price, N. Aronson, J. Brundage, C. Davis, L. Deyton, R. Garner, F. Gordin, D. Henry, W. Holloway, T. Kerkering, R. Luskin-Hawk, J. McNeil, N. Michael, P. Foster Pierce, D. Poretz, S. Ratto-Kim, P. Renzullo, N. Ruiz, K. Sitz, G. Smith, C. Tacket, M. Thompson, E. Tramont, B. Yangco, R. Yarrish, and R. R. Redfield.** 2000. Efficacy testing of recombinant human immunodeficiency virus (HIV) gp160 as a therapeutic vaccine in early-stage HIV-1-infected volunteers. rgp160 Phase II Vaccine Investigators. J Infect Dis **181:**881-9.

33. **Blankson, J., D. Persaud, and R. F. Siliciano.** 1999. Latent reservoirs for HIV-1. Curr Opin Infect Dis **12:**5-11.

34. **Bontrop, R. E., N. Otting, H. Niphuis, R. Noort, V. Teeuwsen, and J. L. Heeney.** 1996. The role of major histocompatibility complex polymorphisms on SIV infection in rhesus macaques. Immunol Lett **51:**35-8.

35. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol **68:**6103-10.

36. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med **3:**205-11.

37. **Boyer, J. D., K. E. Ugen, M. Chattergoon, B. Wang, A. Shah, M. Agadjanyan, M. L. Bagarazzi, A. Javadian, R. Carrano, L. Coney, W. V. Williams, and D. B. Weiner.** 1997. DNA vaccination as anti-human immunodeficiency virus immunotherapy in infected chimpanzees. J Infect Dis **176:**1501-9.

38. **Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek.** 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J Exp Med **200:**749-59.

39. **Buchbinder, S. P., M. H. Katz, N. A. Hessol, P. M. O'Malley, and S. D. Holmberg.** 1994. Long-term HIV-1 infection without immunologic progression. Aids **8:**1123-8.

40. **Buckheit Jr, R. W.** 2004. Understanding HIV resistance, fitness, replication capacity and compensation: targeting viral fitness as a therapeutic strategy. Expert Opinion on Investigational Drugs **13:**933-958.

41. **Buckner, C. M., A. Gettie, R. C. Tan, T. Eshetu, M. Ratterree, J. Blanchard, C. Cheng-Mayer, and J. M. Harouse.** 2002. Infection of macaques with a molecular clone, SHIVSF33A2, provides evidence for tissue specific variants. J Med Primatol **31:**164-70.

42. **Burns, D. P., and R. C. Desrosiers.** 1994. Envelope sequence variation, neutralizing antibodies, and primate lentivirus persistence. Curr Top Microbiol Immunol **188:**185-219.

43. **Burton, G. F., B. F. Keele, J. D. Estes, T. C. Thacker, and S. Gartner.** 2002. Follicular dendritic cell contributions to HIV pathogenesis. Semin Immunol **14:**275-84.

44. **Byrne, J. A., and M. B. Oldstone.** 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. J Virol **51:**682-6.

45. **Calio, R., N. Villani, E. Balestra, F. Sesa, A. Holy, J. Balzarini, E. De Clercq, C. F. Perno, and V. Del Gobbo.** 1994. Enhancement of natural killer activity and interferon induction by different acyclic nucleoside phosphonates. Antiviral Res **23:**77-89.

46. **Cao, Y., L. Qin, L. Zhang, J. Safrit, and D. D. Ho.** 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N Engl J Med **332:**201-8.

47. **Carrington, M., G. W. Nelson, M. P. Martin, T. Kissner, D. Vlahov, J. J. Goedert, R. Kaslow, S. Buchbinder, K. Hoots, and S. J. O'Brien.** 1999. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science **283:**1748-52.

48. **Chackerian, B., L. M. Rudensey, and J. Overbaugh.** 1997. Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. J Virol **71:**7719-27.

49. **Chen, Z. W., A. Craiu, L. Shen, M. J. Kuroda, U. C. Iroku, D. I. Watkins, G. Voss, and N. L. Letvin.** 2000. Simian immunodeficiency virus evades a dominant epitope-specific cytotoxic T lymphocyte response through a mutation resulting in the accelerated dissociation of viral peptide and MHC class I. J Immunol **164:**6474-9.

50. **Chohan, B., D. Lang, M. Sagar, B. Korber, L. Lavreys, B. Richardson, and J. Overbaugh.** 2005. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J Virol **79:**6528-31.

51. **Chopra, M., C. Kendall, Z. Hill, N. Schaay, L. L. Nkonki, and T. M. Doherty.** 2006. 'Nothing new': responses to the introduction of antiretroviral drugs in South Africa. Aids **20:**1975-7.

52. **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387:**183-8.

53. **Chun, T. W., R. T. Davey, Jr., M. Ostrowski, J. Shawn Justement, D. Engel, J. I. Mullins, and A. S. Fauci.** 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. Nat Med **6:**757-61.

54. **Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc Natl Acad Sci U S A **95:**8869-73.

55. **Chun, T. W., L. Stuyver, S. B. Mizell, L. A. Ehler, J. A. Mican, M. Baseler, A. L. Lloyd, M. A. Nowak, and A. S. Fauci.** 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A **94:**13193-7.

56. **Clapham, P. R., and A. McKnight.** 2002. Cell surface receptors, virus entry and tropism of primate lentiviruses. J Gen Virol **83:**1809-29.

57. **Clerici, M., and G. M. Shearer.** 1996. Correlates of protection in HIV infection and the progression of HIV infection to AIDS. Immunol Lett **51:**69-73.

58. **Clerici, M., C. O. Tacket, C. S. Via, D. R. Lucey, S. C. Muluk, R. A. Zajac, R. N. Boswell, J. A. Berzofsky, and G. M. Shearer.** 1991. Immunization with subunit human immunodeficiency virus vaccine generates stronger T helper cell immunity than natural infection. Eur J Immunol **21:**1345-9.

59. **Coffin, J. M.** 1990. Retroviridae and their replication, 2nd ed. Raven Press, New York.

60. **Coffin, J. M.** 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. J Gen Virol **42:**1-26.

61. **Coffin, J. M. F. V.** 1996. Retroviridae: The viruses and their replication, 3 ed. Lippincott-Raven, Philadelphia.

62. **Cohen, O., D. Weissman, and A. S. Fauci. W. E. Paul.** 1999. The Immunopathogenesis of HIV infection. Lippincott-Raven, Philadelphia.

63. **Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr.** 1996. DNA-based immunization by in vivo transfection of dendritic cells. Nat Med **2:**1122-8.

64. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, and et al.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569-71.

65. **Conley, A. J., J. A. Kessler, 2nd, L. J. Boots, J. S. Tung, B. A. Arnold, P. M. Keller, A. R. Shaw, and E. A. Emini.** 1994. Neutralization of divergent human immunodeficiency virus type 1 variants and primary isolates by IAM-41-2F5, an anti-gp41 human monoclonal antibody. Proc Natl Acad Sci U S A **91:**3348-52.

66. **Connor, R. I., and D. D. Ho.** 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. J Virol **68:**4400-8.

67. **Courgnaud, V., W. Saurin, F. Villinger, and P. Sonigo.** 1998. Different evolution of simian immunodeficiency virus in a natural host and a new host. Virology **247:**41-50.

68. **Cranage, M. P., A. M. Whatmore, S. A. Sharpe, N. Cook, N. Polyanskaya, S. Leech, J. D. Smith, E. W. Rud, M. J. Dennis, and G. A. Hall.** 1997. Macaques infected with live attenuated SIVmac are protected against superinfection via the rectal mucosa. Virology **229:**143-54.

69. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763-7.

70. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science **258:**1938-41.

71. **Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers.** 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science **228:**1201-4.

72. **De Cock, K. M.** 2001. Epidemiology and the emergence of human immunodeficiency virus and acquired immune deficiency syndrome. Philos Trans R Soc Lond B Biol Sci **356:**795-8.

73. **Dean, M., M. Carrington, C. Winkler, G. A. Huttley, M. W. Smith, R. Allikmets, J. J. Goedert, S. P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels, and S. J. O'Brien.** 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science **273:**1856-62.

74. **Del Gobbo, V., A. Foli, J. Balzarini, E. De Clercq, E. Balestra, N. Villani, S. Marini, C. F. Perno, and R. Calio.** 1991. Immunomodulatory activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA), a potent anti-HIV nucleotide analogue, on in vivo murine models. Antiviral Res **16:**65-75.

75. **Delwart, E. L., M. P. Busch, M. L. Kalish, J. W. Mosley, and J. I. Mullins.** 1995. Rapid molecular epidemiology of human immunodeficiency virus transmission. AIDS Res Hum Retroviruses **11:**1081-93.

76. **Delwart, E. L., and C. J. Gordon.** 1997. Tracking changes in HIV-1 envelope quasispecies using DNA heteroduplex analysis. Methods **12:**348-54.

77. **Delwart, E. L., J. I. Mullins, P. Gupta, G. H. Learn, Jr., M. Holodniy, D. Katzenstein, B. D. Walker, and M. K. Singh.** 1998. Human immunodeficiency virus type 1 populations in blood and semen. J Virol **72:**617-23.

78. **Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science **262:**1257-61.

79. **Demma, L. J., J. M. Logsdon, T. H. Vanderford, M. B. Feinberg, and S. I. Staprans.** 2005. SIVsm Quasispecies Adaptation to a New Simian Host. PLoS Pathog **1:**e3.

80. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-6.

81. **DeStefano, J. J., R. A. Bambara, and P. J. Fay.** 1994. The mechanism of human immunodeficiency virus reverse transcriptase-catalyzed strand transfer from internal regions of heteropolymeric RNA templates. J Biol Chem **269:**161-8.

82. **DeStefano, J. J., L. M. Mallaber, L. Rodriguez-Rodriguez, P. J. Fay, and R. A. Bambara.** 1992. Requirements for strand transfer between internal regions of heteropolymer templates by human immunodeficiency virus reverse transcriptase. J Virol **66:**6370-8.

83. **Detels, R., A. Munoz, G. McFarlane, L. A. Kingsley, J. B. Margolick, J. Giorgi, L. K. Schrager, and J. P. Phair.** 1998. Effectiveness of potent antiretroviral therapy on time to AIDS and death in men with known HIV infection duration. Multicenter AIDS Cohort Study Investigators. Jama **280:**1497-503.

84. **Devereux, H. L., V. C. Emery, M. A. Johnson, and C. Loveday.** 2001. Replicative fitness in vivo of HIV-1 variants with multiple drug resistance-associated mutations. J Med Virol **65:**218-24.

85. **Devereux, H. L., M. Youle, M. A. Johnson, and C. Loveday.** 1999. Rapid decline in detectability of HIV-1 drug resistance mutations after stopping therapy. Aids **13:**F123-7.

86. **Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux.** 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med **188:**373-86.

87. **Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu.** 1997. DNA vaccines. Annu Rev Immunol **15:**617-48.

88. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667-73.

89. **Dybul, M., M. Daucher, M. A. Jensen, C. W. Hallahan, T. W. Chun, M. Belson, B. Hidalgo, D. C. Nickle, C. Yoder, J. A. Metcalf, R. T. Davey, L. Ehler, D. Kress-Rock, E. Nies-Kraske, S. Liu, J. I. Mullins, and A. S. Fauci.** 2003. Genetic characterization of rebounding human immunodeficiency virus type 1 in plasma during multiple interruptions of highly active antiretroviral therapy. J Virol **77:**3229-37.

90. **Edinger, A. L., J. L. Mankowski, B. J. Doranz, B. J. Margulies, B. Lee, J. Rucker, M. Sharron, T. L. Hoffman, J. F. Berson, M. C. Zink, V. M. Hirsch, J. E. Clements, and R. W. Doms.** 1997. CD4-independent, CCR5-dependent

infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. Proc Natl Acad Sci U S A **94:**14742-7.

91. **Egger, M., B. Hirschel, P. Francioli, P. Sudre, M. Wirz, M. Flepp, M. Rickenbach, R. Malinverni, P. Vernazza, and M. Battegay.** 1997. Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicentre study. Swiss HIV Cohort Study. Bmj **315:**1194-9.

92. **Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes.** 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. DNA Cell Biol **12:**791-7.

93. **Evans, D. T., D. H. O'Connor, P. Jing, J. L. Dzuris, J. Sidney, J. da Silva, T. M. Allen, H. Horton, J. E. Venham, R. A. Rudersdorf, T. Vogel, C. D. Pauza, R. E. Bontrop, R. DeMars, A. Sette, A. L. Hughes, and D. I. Watkins.** 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nat Med **5:**1270-6.

94. **Falo, L. D., Jr., M. Kovacsovics-Bankowski, K. Thompson, and K. L. Rock.** 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nat Med **1:**649-53.

95. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278:**1295-300.

96. **Fischl, M. A., D. D. Richman, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, R. T. Schooley, and et al.** 1987. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. N Engl J Med **317:**185-91.

97. **Fitzgibbon, J. E., R. M. Howell, C. A. Haberzettl, S. J. Sperber, D. J. Gocke, and D. T. Dubin.** 1992. Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine. Antimicrob Agents Chemother **36:**153-7.

98. **Folkvord, J. M., D. M. Anderson, J. Arya, S. MaWhinney, and E. Connick.** 2003. Microanatomic relationships between CD8+ cells and HIV-1-producing cells in human lymphoid tissue in vivo. J Acquir Immune Defic Syndr **32:**469-76.

99. **Forshey, B. M., U. von Schwedler, W. I. Sundquist, and C. Aiken.** 2002. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. J Virol **76:**5667-77.

100. **Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor, and D. I. Watkins.** 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat Med **10:**275-81.

101. **Fuller, D. H., P. A. Rajakumar, L. A. Wilson, A. M. Trichel, J. T. Fuller, T. Shipley, M. S. Wu, K. Weis, C. R. Rinaldo, J. R. Haynes, and M. Murphey-**

**Corb.** 2002. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J Virol **76:**3309-17.

102. **Fuller, D. H., P. A. Rajakumar, M. S. Wu, C. W. McMahon, T. Shipley, J. T. Fuller, A. Bazmi, A. M. Trichel, T. M. Allen, B. Mothe, J. R. Haynes, D. I. Watkins, and M. Murphey-Corb.** 2006. DNA immunization in combination with effective antiretroviral drug therapy controls viral rebound and prevents simian AIDS after treatment is discontinued. Virology.

103. **Galetto, R., V. Giacomoni, M. Veron, and M. Negroni.** 2006. Dissection of a circumscribed recombination hot spot in HIV-1 after a single infectious cycle. J Biol Chem **281:**2711-20.

104. **Gandhi, R. T., A. Wurcel, E. S. Rosenberg, M. N. Johnston, N. Hellmann, M. Bates, M. S. Hirsch, and B. D. Walker.** 2003. Progressive reversion of human immunodeficiency virus type 1 resistance mutations in vivo after transmission of a multiply drug-resistant virus. Clin Infect Dis **37:**1693-8.

105. **Gao, Q., Z. X. Gu, M. A. Parniak, X. G. Li, and M. A. Wainberg.** 1992. In vitro selection of variants of human immunodeficiency virus type 1 resistant to 3'-azido-3'-deoxythymidine and 2',3'-dideoxyinosine. J Virol **66:**12-9.

106. **Gardner, M. B., and P. A. Luciw.** 1988. Simian immunodeficiency viruses and their relationship to the human immunodeficiency viruses. Aids **2 Suppl 1:**S3-10.

107. **Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, K. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Cote, R. L. Rich, D. G. Myszka, and W. I. Sundquist.** 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell **107:**55-65.

108. **George, M. D., E. Reay, S. Sankaran, and S. Dandekar.** 2005. Early antiretroviral therapy for simian immunodeficiency virus infection leads to mucosal CD4+ T-cell restoration and enhanced gene expression regulating mucosal repair and regeneration. J Virol **79:**2709-19.

109. **Godfrey, T. E., S. H. Kim, M. Chavira, D. W. Ruff, R. S. Warren, J. W. Gray, and R. H. Jensen.** 2000. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. J Mol Diagn **2:**84-91.

110. **Goebel, F. D., J. W. Mannhalter, R. B. Belshe, M. M. Eibl, P. J. Grob, V. de Gruttola, P. D. Griffiths, V. Erfle, M. Kunschak, and W. Engl.** 1999. Recombinant gp160 as a therapeutic vaccine for HIV-infection: results of a large randomized, controlled trial. European Multinational IMMUNO AIDS Vaccine Study Group. Aids **13:**1461-8.

111. **Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon.** 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med **305:**1425-31.

112. **Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker.** 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature **412:**334-8.

113. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med **3:**212-7.

114. **Grabar, S., V. Le Moing, C. Goujard, C. Leport, M. D. Kazatchkine, D. Costagliola, and L. Weiss.** 2000. Clinical outcome of patients with HIV-1 infection according to immunologic and virologic response after 6 months of highly active antiretroviral therapy. Ann Intern Med **133:**401-10.

115. **Greenier, J. L., K. K. Van Rompay, D. Montefiori, P. Earl, B. Moss, and M. L. Marthas.** 2005. Simian immunodeficiency virus (SIV) envelope quasispecies transmission and evolution in infant rhesus macaques after oral challenge with uncloned SIVmac251: increased diversity is associated with neutralizing antibodies and improved survival in previously immunized animals. Virol J **2:**11.

116. **Gu, Z., R. S. Fletcher, E. J. Arts, M. A. Wainberg, and M. A. Parniak.** 1994. The K65R mutant reverse transcriptase of HIV-1 cross-resistant to 2', 3'-dideoxycytidine, 2',3'-dideoxy-3'-thiacytidine, and 2',3'-dideoxyinosine shows reduced sensitivity to specific dideoxynucleoside triphosphate inhibitors in vitro. J Biol Chem **269:**28118-22.

117. **Guadalupe, M., S. Sankaran, M. D. George, E. Reay, D. Verhoeven, B. L. Shacklett, J. Flamm, J. Wegelin, T. Prindiville, and S. Dandekar.** 2006. Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. J Virol **80:**8236-47.

118. **Hahn, B. H., G. M. Shaw, K. M. De Cock, and P. M. Sharp.** 2000. AIDS as a zoonosis: scientific and public health implications. Science **287:**607-14.

119. **Halfon, P., J. Durant, P. Clevenbergh, H. Carsenti, L. Celis, H. Khiri, K. De Smet, A. De Brauwer, F. Hulstaert, and P. Dellamonica.** 2003. Kinetics of disappearance of resistance mutations and reappearance of wild-type during structured treatment interruptions. Aids **17:**1351-61.

120. **Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, M. P. Cranage, and A. J. McMichael.** 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. J Virol **73:**7524-32.

121. **Hansen, J. E., O. Lund, N. Tolstrup, A. A. Gooley, K. L. Williams, and S. Brunak.** 1998. NetOglyc: prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. Glycoconj J **15:**115-30.

122. **Harris, R. S., and M. T. Liddament.** 2004. Retroviral restriction by APOBEC proteins. Nat Rev Immunol **4:**868-77.

123. **Hartman, A. L.** 2003. Simian immunodeficiency virus (SIV) production from rhesus macaque CD4+ T lymphocytes *in vitro*: insights into the host factors controlling the rate of progression to AIDS *in vivo*. Dissertation. University of Pittsburgh.

124. **Heeney, J. L., C. van Els, P. de Vries, P. ten Haaft, N. Otting, W. Koornstra, J. Boes, R. Dubbes, H. Niphuis, M. Dings, M. Cranage, S. Norley, M. Jonker,**

**R. E. Bontrop, and A. Osterhaus.** 1994. Major histocompatibility complex class I-associated vaccine protection from simian immunodeficiency virus-infected peripheral blood cells. J Exp Med **180:**769-74.

125. **Hel, Z., D. Venzon, M. Poudyal, W. P. Tsai, L. Giuliani, R. Woodward, C. Chougnet, G. Shearer, J. D. Altman, D. Watkins, N. Bischofberger, A. Abimiku, P. Markham, J. Tartaglia, and G. Franchini.** 2000. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. Nat Med **6:**1140-6.

126. **Hirsch, V., D. Adger-Johnson, B. Campbell, S. Goldstein, C. Brown, W. R. Elkins, and D. C. Montefiori.** 1997. A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. J Virol **71:**1608-20.

127. **Hirsch, V. M., and P. R. Johnson.** 1994. Pathogenic diversity of simian immunodeficiency viruses. Virus Res **32:**183-203.

128. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123-6.

129. **Holterman, L., R. Dubbes, J. Mullins, G. Learn, H. Niphuis, W. Koornstra, G. Koopman, E. M. Kuhn, A. Wade-Evans, B. Rosenwirth, J. Haaijman, and J. Heeney.** 2001. Characteristics of a pathogenic molecular clone of an end-stage serum-derived variant of simian immunodeficiency virus (SIV(F359)). J Virol **75:**9328-38.

130. **Holterman, L., H. Niphuis, P. J. ten Haaft, J. Goudsmit, G. Baskin, and J. L. Heeney.** 1999. Specific passage of simian immunodeficiency virus from end-stage disease results in accelerated progression to AIDS in rhesus macaques. J Gen Virol **80 (Pt 12):**3089-97.

131. **Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins.** 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J Virol **76:**7187-202.

132. **Hu, S. L., K. Abrams, L. Misher, V. Stallard, P. Moran, J. M. Zarling, A. J. Langlois, L. Kuller, W. R. Morton, and R. E. Benveniste.** 1992. Evaluation of protective efficacy of recombinant subunit vaccines against simian immunodeficiency virus infection of macaques. J Med Primatol **21:**119-25.

133. **Huang, Y., W. A. Paxton, S. M. Wolinsky, A. U. Neumann, L. Zhang, T. He, S. Kang, D. Ceradini, Z. Jin, K. Yazdanbakhsh, K. Kunstman, D. Erickson, E. Dragon, N. R. Landau, J. Phair, D. D. Ho, and R. A. Koup.** 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med **2:**1240-3.

134. **Huang, Y., L. Zhang, and D. D. Ho.** 1995. Biological characterization of nef in long-term survivors of human immunodeficiency virus type 1 infection. J Virol **69:**8142-6.

135. **Huang, Y., L. Zhang, and D. D. Ho.** 1995. Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. J Virol **69:**93-100.

136. **Hutchinson, A. B., P. G. Farnham, H. D. Dean, D. U. Ekwueme, C. Del Rio, L. Kamimoto, and S. E. Kellerman.** 2006. The Economic Burden of HIV in the United States in the Era of Highly Active Antiretroviral Therapy: Evidence of Continuing Racial and Ethnic Differences. J Acquir Immune Defic Syndr.

137. **Hymes, K. B., T. Cheung, J. B. Greene, N. S. Prose, A. Marcus, H. Ballard, D. C. William, and L. J. Laubenstein.** 1981. Kaposi's sarcoma in homosexual men-a report of eight cases. Lancet **2:**598-600.

138. **Jacque, J. M., and M. Stevenson.** 2006. The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. Nature **441:**641-5.

139. **Jakubik, J. J., M. Saifuddin, D. M. Takefman, and G. T. Spear.** 2000. Immune complexes containing human immunodeficiency virus type 1 primary isolates bind to lymphoid tissue B lymphocytes and are infectious for T lymphocytes. J Virol **74:**552-5.

140. **Jarmuz, A., A. Chester, J. Bayliss, J. Gisbourne, I. Dunham, J. Scott, and N. Navaratnam.** 2002. An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. Genomics **79:**285-96.

141. **Jetzt, A. E., H. Yu, G. J. Klarmann, Y. Ron, B. D. Preston, and J. P. Dougherty.** 2000. High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol **74:**1234-40.

142. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med **189:**991-8.

143. **Joag, S. V., Z. Li, L. Foresman, D. M. Pinson, E. B. Stephens, R. Raghavan, J. F. Nave, P. Casara, and O. Narayan.** 1997. Early treatment with 9-(2-phosphonylmethoxyethyl)adenine reduces virus burdens for a prolonged period in SIV-infected rhesus macaques. AIDS Res Hum Retroviruses **13:**241-6.

144. **Jones, N. A., X. Wei, D. R. Flower, M. Wong, F. Michor, M. S. Saag, B. H. Hahn, M. A. Nowak, G. M. Shaw, and P. Borrow.** 2004. Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. J Exp Med **200:**1243-56.

145. **Joos, B., A. Trkola, M. Fischer, H. Kuster, P. Rusert, C. Leemann, J. Boni, A. Oxenius, D. A. Price, R. E. Phillips, J. K. Wong, B. Hirschel, R. Weber, and H. F. Gunthard.** 2005. Low human immunodeficiency virus envelope diversity correlates with low in vitro replication capacity and predicts spontaneous control of plasma viremia after treatment interruptions. J Virol **79:**9026-37.

146. **Kaufmann, D., G. Pantaleo, P. Sudre, and A. Telenti.** 1998. CD4-cell count in HIV-1-infected individuals remaining viraemic with highly active antiretroviral therapy (HAART). Swiss HIV Cohort Study. Lancet **351:**723-4.

147. **Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw.** 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. J Virol **72:**10180-8.

148. **Kijak, G. H., V. Simon, P. Balfe, J. Vanderhoeven, S. E. Pampuro, C. Zala, C. Ochoa, P. Cahn, M. Markowitz, and H. Salomon.** 2002. Origin of human immunodeficiency virus type 1 quasispecies emerging after antiretroviral treatment interruption in patients with therapeutic failure. J Virol **76:**7000-9.

149. **Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J Virol **63:**3708-13.

150. **Kimata, J. T., L. Kuller, D. B. Anderson, P. Dailey, and J. Overbaugh.** 1999. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nat Med **5:**535-41.

151. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med **332:**228-32.

152. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature **312:**767-8.

153. **Knapp, L. A., E. Lehmann, M. S. Piekarczyk, J. A. Urvater, and D. I. Watkins.** 1997. A high frequency of Mamu-A*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. Tissue Antigens **50:**657-61.

154. **Kobayashi, M., H. Igarashi, A. Takeda, M. Kato, and T. Matano.** 2005. Reversion in vivo after inoculation of a molecular proviral DNA clone of simian immunodeficiency virus with a cytotoxic-T-lymphocyte escape mutation. J Virol **79:**11529-32.

155. **Koch, M., M. Pancera, P. D. Kwong, P. Kolchinsky, C. Grundner, L. Wang, W. A. Hendrickson, J. Sodroski, and R. Wyatt.** 2003. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. Virology **313:**387-400.

156. **Kodama, T., K. Mori, T. Kawahara, D. J. Ringler, and R. C. Desrosiers.** 1993. Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. J Virol **67:**6522-34.

157. **Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B. H. Hahn, S. Wolinsky, and T. Bhattacharya.** 2000. Timing the ancestor of the HIV-1 pandemic strains. Science **288:**1789-96.

158. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune

responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol **68:**4650-5.

159. **Kowalczyk, D. W., and H. C. Ertl.** 1999. Immune responses to DNA vaccines. Cell Mol Life Sci **55:**751-70.

160. **Kundu, S. K., D. Katzenstein, L. E. Moses, and T. C. Merigan.** 1992. Enhancement of human immunodeficiency virus (HIV)-specific CD4+ and CD8+ cytotoxic T-lymphocyte activities in HIV-infected asymptomatic patients given recombinant gp160 vaccine. Proc Natl Acad Sci U S A **89:**11204-8.

161. **Kuroda, M. J., J. E. Schmitz, A. Seth, R. S. Veazey, C. E. Nickerson, M. A. Lifton, P. J. Dailey, M. A. Forman, P. Racz, K. Tenner-Racz, and N. L. Letvin.** 2000. Simian immunodeficiency virus-specific cytotoxic T lymphocytes and cell-associated viral RNA levels in distinct lymphoid compartments of SIVmac-infected rhesus monkeys. Blood **96:**1474-9.

162. **Kuwata, T., H. Dehghani, C. R. Brown, R. Plishka, A. Buckler-White, T. Igarashi, J. Mattapallil, M. Roederer, and V. M. Hirsch.** 2006. Infectious molecular clones from a simian immunodeficiency virus-infected rapid-progressor (RP) macaque: evidence of differential selection of RP-specific envelope mutations in vitro and in vivo. J Virol **80:**1463-75.

163. **Lane, H. C., J. M. Depper, W. C. Greene, G. Whalen, T. A. Waldmann, and A. S. Fauci.** 1985. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. Evidence for a selective defect in soluble antigen recognition. N Engl J Med **313:**79-84.

164. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731-4.

165. **Learmont, J., B. Tindall, L. Evans, A. Cunningham, P. Cunningham, J. Wells, R. Penny, J. Kaldor, and D. A. Cooper.** 1992. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. Lancet **340:**863-7.

166. **Lecossier, D., F. Bouchonnet, F. Clavel, and A. J. Hance.** 2003. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science **300:**1112.

167. **Lehmann-Grube, F., D. Moskophidis, and J. Lohler.** 1988. Recovery from acute virus infection. Role of cytotoxic T lymphocytes in the elimination of lymphocytic choriomeningitis virus from spleens of mice. Ann N Y Acad Sci **532:**238-56.

168. **Leitner, T., E. Halapi, G. Scarlatti, P. Rossi, J. Albert, E. M. Fenyo, and M. Uhlen.** 1993. Analysis of heterogeneous viral populations by direct DNA sequencing. Biotechniques **15:**120-7.

169. **Lemey, P., O. G. Pybus, B. Wang, N. K. Saksena, M. Salemi, and A. M. Vandamme.** 2003. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci U S A **100:**6588-92.

170. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V.**

**Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat Med **10:**282-9.

171. **Letvin, N. L., D. C. Montefiori, Y. Yasutomi, H. C. Perry, M. E. Davies, C. Lekutis, M. Alroy, D. C. Freed, C. I. Lord, L. K. Handt, M. A. Liu, and J. W. Shiver.** 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proc Natl Acad Sci U S A **94:**9378-83.

172. **Letvin, N. L., J. E. Schmitz, H. L. Jordan, A. Seth, V. M. Hirsch, K. A. Reimann, and M. J. Kuroda.** 1999. Cytotoxic T lymphocytes specific for the simian immunodeficiency virus. Immunol Rev **170:**127-34.

173. **Levy, D. N., G. M. Aldrovandi, O. Kutsch, and G. M. Shaw.** 2004. Dynamics of HIV-1 recombination in its natural target cells. Proc Natl Acad Sci U S A **101:**4204-9.

174. **Lifson, A. R., G. W. Rutherford, and H. W. Jaffe.** 1988. The natural history of human immunodeficiency virus infection. J Infect Dis **158:**1360-7.

175. **Lifson, J. D., M. A. Nowak, S. Goldstein, J. L. Rossio, A. Kinter, G. Vasquez, T. A. Wiltrout, C. Brown, D. Schneider, L. Wahl, A. L. Lloyd, J. Williams, W. R. Elkins, A. S. Fauci, and V. M. Hirsch.** 1997. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J Virol **71:**9508-14.

176. **Lifson, J. D., M. Piatak, Jr., A. N. Cline, J. L. Rossio, J. Purcell, I. Pandrea, N. Bischofberger, J. Blanchard, and R. S. Veazey.** 2003. Transient early post-

inoculation anti-retroviral treatment facilitates controlled infection with sparing of CD4+ T cells in gut-associated lymphoid tissues in SIVmac239-infected rhesus macaques, but not resistance to rechallenge. J Med Primatol **32:**201-10.

177. **Lifson, J. D., J. L. Rossio, R. Arnaout, L. Li, T. L. Parks, D. K. Schneider, R. F. Kiser, V. J. Coalter, G. Walsh, R. J. Imming, B. Fisher, B. M. Flynn, N. Bischofberger, M. Piatak, Jr., V. M. Hirsch, M. A. Nowak, and D. Wodarz.** 2000. Containment of simian immunodeficiency virus infection: cellular immune responses and protection from rechallenge following transient postinoculation antiretroviral treatment. J Virol **74:**2584-93.

178. **Lisziewicz, J., J. Trocio, J. Xu, L. Whitman, A. Ryder, N. Bakare, M. G. Lewis, W. Wagner, A. Pistorio, S. Arya, and F. Lori.** 2005. Control of viral rebound through therapeutic immunization with DermaVir. Aids **19:**35-43.

179. **Little, S. J., A. R. McLean, C. A. Spina, D. D. Richman, and D. V. Havlir.** 1999. Viral dynamics of acute HIV-1 infection. J Exp Med **190:**841-50.

180. **Louie, M., C. Hogan, A. Hurley, V. Simon, C. Chung, N. Padte, P. Lamy, J. Flaherty, D. Coakley, M. Di Mascio, A. S. Perelson, and M. Markowitz.** 2003. Determining the antiviral activity of tenofovir disoproxil fumarate in treatment-naive chronically HIV-1-infected individuals. Aids **17:**1151-6.

181. **Lukashov, V. V., C. L. Kuiken, and J. Goudsmit.** 1995. Intrahost human immunodeficiency virus type 1 evolution is related to length of the immunocompetent period. J Virol **69:**6911-6.

182. **MacDonald, K. S., K. R. Fowke, J. Kimani, V. A. Dunand, N. J. Nagelkerke, T. B. Ball, J. Oyugi, E. Njagi, L. K. Gaur, R. C. Brunham, J. Wade, M. A.**

**Luscher, P. Krausa, S. Rowland-Jones, E. Ngugi, J. J. Bwayo, and F. A. Plummer.** 2000. Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection. J Infect Dis **181:**1581-9.

183. **MacGregor, R. R., J. D. Boyer, K. E. Ugen, P. Tebas, T. J. Higgins, Y. Baine, R. B. Ciccarelli, R. S. Ginsberg, and D. B. Weiner.** 2005. Plasmid vaccination of stable HIV-positive subjects on antiviral treatment results in enhanced CD8 T-cell immunity and increased control of viral "blips". Vaccine **23:**2066-73.

184. **Magierowska, M., F. Bernardin, S. Garg, S. Staprans, M. D. Miller, K. K. Van Rompay, and E. L. Delwart.** 2004. Highly uneven distribution of tenofovir-selected simian immunodeficiency virus in different anatomical sites of rhesus macaques. J Virol **78:**2434-44.

185. **Malaspina, A., S. Moir, D. C. Nickle, E. T. Donoghue, K. M. Ogwaro, L. A. Ehler, S. Liu, J. A. Mican, M. Dybul, T. W. Chun, J. I. Mullins, and A. S. Fauci.** 2002. Human immunodeficiency virus type 1 bound to B cells: relationship to virus replicating in CD4+ T cells and circulating in plasma. J Virol **76:**8855-63.

186. **Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono.** 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **424:**99-103.

187. **Mann, J. M.** 1987. Current Topics in AIDS, vol. 2. Wiley, New York.

188. **Mansky, L. M., and H. M. Temin.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol **69:**5087-94.

189. **Martin, K. A., R. Wyatt, M. Farzan, H. Choe, L. Marcon, E. Desjardins, J. Robinson, J. Sodroski, C. Gerard, and N. P. Gerard.** 1997. CD4-independent binding of SIV gp120 to rhesus CCR5. Science **278:**1470-3.

190. **Martin, L. N., M. Murphey-Corb, K. F. Soike, B. Davison-Fairburn, and G. B. Baskin.** 1993. Effects of initiation of 3'-azido,3'-deoxythymidine (zidovudine) treatment at different times after infection of rhesus monkeys with simian immunodeficiency virus. J Infect Dis **168:**825-35.

191. **Marx, P. A., N. C. Pedersen, N. W. Lerche, K. G. Osborn, L. J. Lowenstine, A. A. Lackner, D. H. Maul, H. S. Kwang, J. D. Kluge, C. P. Zaiss, and et al.** 1986. Prevention of simian acquired immune deficiency syndrome with a formalin-inactivated type D retrovirus vaccine. J Virol **60:**431-5.

192. **Mascola, J. R., M. G. Lewis, T. C. VanCott, G. Stiegler, H. Katinger, M. Seaman, K. Beaudry, D. H. Barouch, B. Korioth-Schmitz, G. Krivulka, A. Sambor, B. Welcher, D. C. Douek, D. C. Montefiori, J. W. Shiver, P. Poignard, D. R. Burton, and N. L. Letvin.** 2003. Cellular immunity elicited by human immunodeficiency virus type 1/ simian immunodeficiency virus DNA vaccination does not augment the sterile protection afforded by passive infusion of neutralizing antibodies. J Virol **77:**10348-56.

193. **Matloubian, M., R. J. Concepcion, and R. Ahmed.** 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol **68:**8056-63.

194. **Matzinger, P.** 1994. Tolerance, danger, and the extended family. Annu Rev Immunol **12:**991-1045.

195. **McNicholl, J. M., and K. T. Cuenco.** 1999. Host genes and infectious diseases. HIV, other pathogens, and a public health perspective. Am J Prev Med **16:**141-54.

196. **Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz.** 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med **200:**761-70.

197. **Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley.** 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **272:**1167-70.

198. **Meyer, L., M. Magierowska, J. B. Hubert, C. Rouzioux, C. Deveau, F. Sanson, P. Debre, J. F. Delfraissy, and I. Theodorou.** 1997. Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group. Aids **11:**F73-8.

199. **Michael, N. L.** 1999. Host genetic influences on HIV-1 pathogenesis. Curr Opin Immunol **11:**466-74.

200. **Michael, N. L., G. Chang, L. A. d'Arcy, P. K. Ehrenberg, R. Mariani, M. P. Busch, D. L. Birx, and D. H. Schwartz.** 1995. Defective accessory genes in a human immunodeficiency virus type 1-infected long-term survivor lacking recoverable virus. J Virol **69:**4228-36.

201. **Michael, N. L., G. Chang, L. G. Louie, J. R. Mascola, D. Dondero, D. L. Birx, and H. W. Sheppard.** 1997. The role of viral phenotype and CCR-5 gene defects in HIV-1 transmission and disease progression. Nat Med **3:**338-40.

202. **Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci U S A **97:**2709-14.

203. **Miyazawa, K., A. Mori, K. Yamamoto, and H. Okudaira.** 1998. Constitutive transcription of the human interleukin-6 gene by rheumatoid synoviocytes: spontaneous activation of NF-kappaB and CBF1. Am J Pathol **152:**793-803.

204. **Mohri, H., S. Bonhoeffer, S. Monard, A. S. Perelson, and D. D. Ho.** 1998. Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. Science **279:**1223-7.

205. **Montefiori, D. C., I. Y. Zhou, B. Barnes, D. Lake, E. M. Hersh, Y. Masuho, and L. B. Lefkowitz, Jr.** 1991. Homotypic antibody responses to fresh clinical isolates of human immunodeficiency virus. Virology **182:**635-43.

206. **Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal.** 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science **296:**1439-43.

207. **Mor, G., M. Singla, A. D. Steinberg, S. L. Hoffman, K. Okuda, and D. M. Klinman.** 1997. Do DNA vaccines induce autoimmune disease? Hum Gene Ther **8:**293-300.

208. **Morgan, D., C. Mahe, B. Mayanja, J. M. Okongo, R. Lubega, and J. A. Whitworth.** 2002. HIV-1 infection in rural Africa: is there a difference in median

time to AIDS and survival compared with that in industrialized countries? Aids **16:**597-603.

209. **Moss, R. B., W. K. Giermakowska, J. R. Savary, G. Theofan, A. E. Daigle, S. P. Richieri, F. C. Jensen, and D. J. Carlo.** 1998. A primer on HIV type 1-specific immune function and REMUNE. AIDS Res Hum Retroviruses **14 Suppl 2:**S167-75.

210. **Mothe, B. R., H. Horton, D. K. Carter, T. M. Allen, M. E. Liebl, P. Skinner, T. U. Vogel, S. Fuenger, K. Vielhuber, W. Rehrauer, N. Wilson, G. Franchini, J. D. Altman, A. Haase, L. J. Picker, D. B. Allison, and D. I. Watkins.** 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. J Virol **76:**875-84.

211. **Mothe, B. R., J. Weinfurter, C. Wang, W. Rehrauer, N. Wilson, T. M. Allen, D. B. Allison, and D. I. Watkins.** 2003. Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol **77:**2736-40.

212. **Munoz, A., A. J. Kirby, Y. D. He, J. B. Margolick, B. R. Visscher, C. R. Rinaldo, R. A. Kaslow, and J. P. Phair.** 1995. Long-term survivors with HIV-1 infection: incubation period and longitudinal patterns of CD4+ lymphocytes. J Acquir Immune Defic Syndr Hum Retrovirol **8:**496-505.

213. **Murphey-Corb, M., L. N. Martin, B. Davison-Fairburn, R. C. Montelaro, M. Miller, M. West, S. Ohkawa, G. B. Baskin, J. Y. Zhang, S. Putney, and et al.**

1990. A formalin inactivated whole SIV vaccine and a glycoprotein-enriched subunit vaccine confers protection against experimental challenge with pathogenic live SIV in rhesus monkeys. Dev Biol Stand **72:**273-85.

214. **Murphey-Corb, M., L. N. Martin, S. R. Rangan, G. B. Baskin, B. J. Gormus, R. H. Wolf, W. A. Andes, M. West, and R. C. Montelaro.** 1986. Isolation of an HTLV-III-related retrovirus from macaques with simian AIDS and its possible origin in asymptomatic mangabeys. Nature **321:**435-7.

215. **Murphey-Corb, M., L. A. Wilson, A. M. Trichel, D. E. Roberts, K. Xu, S. Ohkawa, B. Woodson, R. Bohm, and J. Blanchard.** 1999. Selective induction of protective MHC class I-restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. J Immunol **162:**540-9.

216. **Murry, J. P., J. Higgins, T. B. Matthews, V. Y. Huang, K. K. Van Rompay, N. C. Pedersen, and T. W. North.** 2003. Reversion of the M184V mutation in simian immunodeficiency virus reverse transcriptase is selected by tenofovir, even in the presence of lamivudine. J Virol **77:**1120-30.

217. **Naesens, L., J. Balzarini, and E. De Clercq.** 1991. Single-dose administration of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) in the prophylaxis of retrovirus infection in vivo. Antiviral Res **16:**53-64.

218. **National Aids Trust**. **nat.org.uk.** Statistics, International Summary. Updated November 2006.

219. **Nath, B. M., K. E. Schumann, and J. D. Boyer.** 2000. The chimpanzee and other non-human-primate models in HIV-1 vaccine research. Trends Microbiol **8:**426-31.

220. **Negroni, M., and H. Buc.** 2001. Mechanisms of retroviral recombination. Annu Rev Genet **35:**275-302.

221. **Nichols, W. W., B. J. Ledwith, S. V. Manam, and P. J. Troilo.** 1995. Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci **772:**30-9.

222. **Nilsson, C., B. Makitalo, R. Thorstensson, S. Norley, D. Binninger-Schinzel, M. Cranage, E. Rud, G. Biberfeld, and P. Putkonen.** 1998. Live attenuated simian immunodeficiency virus (SIV)mac in macaques can induce protection against mucosal infection with SIVsm. Aids **12:**2261-70.

223. **Nishimura, Y., T. Igarashi, N. L. Haigwood, R. Sadjadpour, O. K. Donau, C. Buckler, R. J. Plishka, A. Buckler-White, and M. A. Martin.** 2003. Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. Proc Natl Acad Sci U S A **100:**15131-6.

224. **Noe, A., J. Plum, and C. Verhofstede.** 2005. The latent HIV-1 reservoir in patients undergoing HAART: an archive of pre-HAART drug resistance. J Antimicrob Chemother **55:**410-2.

225. **North, T. W., K. K. Van Rompay, J. Higgins, T. B. Matthews, D. A. Wadford, N. C. Pedersen, and R. F. Schinazi.** 2005. Suppression of virus load by highly active antiretroviral therapy in rhesus macaques infected with a

recombinant simian immunodeficiency virus containing reverse transcriptase from human immunodeficiency virus type 1. J Virol **79:**7349-54.

226. **Novembre, F. J., M. Saucier, D. C. Anderson, S. A. Klumpp, S. P. O'Neil, C. R. Brown, 2nd, C. E. Hart, P. C. Guenthner, R. B. Swenson, and H. M. McClure.** 1997. Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol **71:**4086-91.

227. **O'Brien, S. J., G. W. Nelson, C. A. Winkler, and M. W. Smith.** 2000. Polygenic and multifactorial disease gene association in man: Lessons from AIDS. Annu Rev Genet **34:**563-591.

228. **O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat Med **8:**493-9.

229. **O'Connor, D. H., A. B. McDermott, K. C. Krebs, E. J. Dodds, J. E. Miller, E. J. Gonzalez, T. J. Jacoby, L. Yant, H. Piontkivska, R. Pantophlet, D. R. Burton, W. M. Rehrauer, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2004. A dominant role for CD8+-T-lymphocyte selection in simian immunodeficiency virus sequence variation. J Virol **78:**14012-22.

230. **O'Connor, D. H., B. R. Mothe, J. T. Weinfurter, S. Fuenger, W. M. Rehrauer, P. Jing, R. R. Rudersdorf, M. E. Liebl, K. Krebs, J. Vasquez, E. Dodds, J. Loffredo, S. Martin, A. B. McDermott, T. M. Allen, C. Wang, G. G. Doxiadis, D. C. Montefiori, A. Hughes, D. R. Burton, D. B. Allison, S. M.**

**Wolinsky, R. Bontrop, L. J. Picker, and D. I. Watkins.** 2003. Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. J Virol **77:**9029-40.

231. **Overbaugh, J., L. M. Rudensey, M. D. Papenhausen, R. E. Benveniste, and W. R. Morton.** 1991. Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. J Virol **65:**7025-31.

232. **Pal, R., D. Venzon, N. L. Letvin, S. Santra, D. C. Montefiori, N. R. Miller, E. Tryniszewska, M. G. Lewis, T. C. VanCott, V. Hirsch, R. Woodward, A. Gibson, M. Grace, E. Dobratz, P. D. Markham, Z. Hel, J. Nacsa, M. Klein, J. Tartaglia, and G. Franchini.** 2002. ALVAC-SIV-gag-pol-env-based vaccination and macaque major histocompatibility complex class I (A*01) delay simian immunodeficiency virus SIVmac-induced immunodeficiency. J Virol **76:**292-302.

233. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med **338:**853-60.

234. **Parren, P. W., M. C. Gauduin, R. A. Koup, P. Poignard, Q. J. Sattentau, P. Fisicaro, and D. R. Burton.** 1997. Erratum to "Relevance of the antibody response against human immunodeficiency virus type 1 envelope to vaccine design". Immunol Lett **58:**125-32.

235. **Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau.** 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. Aids **13 Suppl A:**S137-62.

236. **Peng, B., R. Voltan, L. Lim, Y. Edghill-Smith, S. Phogat, D. S. Dimitrov, K. Arora, M. Leno, S. Than, R. Woodward, P. D. Markham, M. Cranage, and M. Robert-Guroff.** 2002. Rhesus macaque resistance to mucosal simian immunodeficiency virus infection is associated with a postentry block in viral replication. J Virol **76:**6016-26.

237. **Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard, and D. D. Ho.** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science **271:**1582-6.

238. **Petry, H., K. Pekrun, G. Hunsmann, E. Jurkiewicz, and W. Luke.** 2000. Naturally occurring V1-env region variants mediate simian immunodeficiency virus SIVmac escape from high-titer neutralizing antibodies induced by a protective subunit vaccine. J Virol **74:**11145-52.

239. **Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain.** 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med **188:**1075-82.

240. **Poss, M., H. L. Martin, J. K. Kreiss, L. Granville, B. Chohan, P. Nyange, K. Mandaliya, and J. Overbaugh.** 1995. Diversity in virus populations from genital secretions and peripheral blood from women recently infected with human immunodeficiency virus type 1. J Virol **69:**8118-22.

241. **Potvin, D. M., D. W. Metzger, W. T. Lee, D. N. Collins, and A. I. Ramsingh.** 2003. Exogenous interleukin-12 protects against lethal infection with coxsackievirus B4. J Virol **77:**8272-9.

242. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci U S A **94:**1890-5.

243. **Quinnan, G. V., Jr., X. F. Yu, M. G. Lewis, P. F. Zhang, G. Sutter, P. Silvera, M. Dong, A. Choudhary, P. T. Sarkis, P. Bouma, Z. Zhang, D. C. Montefiori, T. C. Vancott, and C. C. Broder.** 2005. Protection of rhesus monkeys against infection with minimally pathogenic simian-human immunodeficiency virus: correlations with neutralizing antibodies and cytotoxic T cells. J Virol **79:**3358-69.

244. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat Med **4:**679-84.

245. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A **100:**4144-9.

246. **Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure.** 1999. Neutralizing antibody-independent

containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nat Med **5:**526-34.

247. **Rowland-Jones, S., S. Pinheiro, and R. Kaul.** 2001. New insights into host factors in HIV-1 pathogenesis. Cell **104:**473-6.

248. **Rowland-Jones, S., J. Sutton, K. Ariyoshi, T. Dong, F. Gotch, S. McAdam, D. Whitby, S. Sabally, A. Gallimore, T. Corrah, and et al.** 1995. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. Nat Med **1:**59-64.

249. **Rudensey, L. M., J. T. Kimata, E. M. Long, B. Chackerian, and J. Overbaugh.** 1998. Changes in the extracellular envelope glycoprotein of variants that evolve during the course of simian immunodeficiency virus SIVMne infection affect neutralizing antibody recognition, syncytium formation, and macrophage tropism but not replication, cytopathicity, or CCR-5 coreceptor recognition. J Virol **72:**209-17.

250. **Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, and et al.** 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc Natl Acad Sci U S A **85:**3198-202.

251. **Rybarczyk, B. J., D. Montefiori, P. R. Johnson, A. West, R. E. Johnston, and R. Swanstrom.** 2004. Correlation between env V1/V2 region diversification and neutralizing antibodies during primary infection by simian immunodeficiency virus sm in rhesus macaques. J Virol **78:**3561-71.

252. **Rychert, J., N. Lacour, and A. M. Amedee.** 2006. Genetic analysis of simian immunodeficiency virus expressed in milk and selectively transmitted through breastfeeding. J Virol **80:**3721-31.

253. **Salmon, J. K., C. A. Armstrong, and J. C. Ansel.** 1994. The skin as an immune organ. West J Med **160:**146-52.

254. **Sandberg, J. K., N. M. Fast, K. A. Jordan, S. N. Furlan, J. D. Barbour, G. Fennelly, J. Dobroszycki, H. M. Spiegel, A. Wiznia, M. G. Rosenberg, and D. F. Nixon.** 2003. HIV-specific CD8+ T cell function in children with vertically acquired HIV-1 infection is critically influenced by age and the state of the CD4+ T cell compartment. J Immunol **170:**4403-10.

255. **Sawai, E. T., M. S. Hamza, M. Ye, K. E. Shaw, and P. A. Luciw.** 2000. Pathogenic conversion of live attenuated simian immunodeficiency virus vaccines is associated with expression of truncated Nef. J Virol **74:**2038-45.

256. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science **283:**857-60.

257. **Schneider, M. F., S. J. Gange, C. M. Williams, K. Anastos, R. M. Greenblatt, L. Kingsley, R. Detels, and A. Munoz.** 2005. Patterns of the hazard of death after AIDS through the evolution of antiretroviral therapy: 1984-2004. Aids **19:**2009-18.

258. **Schooley, R. T., P. Ruane, R. A. Myers, G. Beall, H. Lampiris, D. Berger, S. S. Chen, M. D. Miller, E. Isaacson, and A. K. Cheng.** 2002. Tenofovir DF in antiretroviral-experienced patients: results from a 48-week, randomized, double-blind study. Aids **16:**1257-63.

259. **Sei, Y., P. H. Tsang, F. N. Chu, I. Wallace, J. P. Roboz, P. S. Sarin, and J. G. Bekesi.** 1989. Inverse relationship between HIV-1 p24 antigenemia, anti-p24 antibody and neutralizing antibody response in all stages of HIV-1 infection. Immunol Lett **20:**223-30.

260. **Sei, Y., P. H. Tsang, J. P. Roboz, P. S. Sarin, J. I. Wallace, and J. G. Bekesi.** 1988. Neutralizing antibodies as a prognostic indicator in the progression of acquired immune deficiency syndrome (AIDS)-related disorders: a double-blind study. J Clin Immunol **8:**464-72.

261. **Seman, A. L., W. F. Pewen, L. F. Fresh, L. N. Martin, and M. Murphey-Corb.** 2000. The replicative capacity of rhesus macaque peripheral blood mononuclear cells for simian immunodeficiency virus in vitro is predictive of the rate of progression to AIDS in vivo. J Gen Virol **81:**2441-9.

262. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I. Mullins.** 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol **73:**10489-502.

263. **Sharp, P. M., E. Bailes, F. Gao, B. E. Beer, V. M. Hirsch, and B. H. Hahn.** 2000. Origins and evolution of AIDS viruses: estimating the time-scale. Biochem Soc Trans **28:**275-82.

264. **Sharp, P. M., E. Bailes, D. L. Robertson, F. Gao, and B. H. Hahn.** 1999. Origins and evolution of AIDS viruses. Biol Bull **196:**338-42.

265. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646-50.

266. **Shen, A., M. C. Zink, J. L. Mankowski, K. Chadwick, J. B. Margolick, L. M. Carruth, M. Li, J. E. Clements, and R. F. Siliciano.** 2003. Resting CD4+ T lymphocytes but not thymocytes provide a latent viral reservoir in a simian immunodeficiency virus-Macaca nemestrina model of human immunodeficiency virus type 1-infected patients on highly active antiretroviral therapy. J Virol **77:**4938-49.

267. **Sheppard, H. W., W. Lang, M. S. Ascher, E. Vittinghoff, and W. Winkelstein.** 1993. The characterization of non-progressors: long-term HIV-1 infection with stable CD4+ T-cell levels. Aids **7:**1159-66.

268. **Shin, H. D., C. Winkler, J. C. Stephens, J. Bream, H. Young, J. J. Goedert, T. R. O'Brien, D. Vlahov, S. Buchbinder, J. Giorgi, C. Rinaldo, S. Donfield, A. Willoughby, S. J. O'Brien, and M. W. Smith.** 2000. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. Proc Natl Acad Sci U S A **97:**14467-72.

269. **Silvestri, G., A. Fedanov, S. Germon, N. Kozyr, W. J. Kaiser, D. A. Garber, H. McClure, M. B. Feinberg, and S. I. Staprans.** 2005. Divergent host responses during primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. J Virol **79:**4043-54.

270. **Sluis-Cremer, N., D. Arion, and M. A. Parniak.** 2000. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). Cell Mol Life Sci **57:**1408-22.

271. **Smit-McBride, Z., J. J. Mattapallil, M. McChesney, D. Ferrick, and S. Dandekar.** 1998. Gastrointestinal T lymphocytes retain high potential for cytokine responses but have severe CD4(+) T-cell depletion at all stages of simian immunodeficiency virus infection compared to peripheral lymphocytes. J Virol **72:**6646-56.

272. **Soudeyns, H., S. Paolucci, C. Chappey, M. B. Daucher, C. Graziosi, M. Vaccarezza, O. J. Cohen, A. S. Fauci, and G. Pantaleo.** 1999. Selective pressure exerted by immunodominant HIV-1-specific cytotoxic T lymphocyte responses during primary infection drives genetic variation restricted to the cognate epitope. Eur J Immunol **29:**3629-35.

273. **Spring, M., C. Stahl-Hennig, N. Stolte, N. Bischofberger, J. Heeney, P. ten Haaft, K. Tenner-Racz, P. Racz, D. Lorenzen, G. Hunsmann, and U. Dittmer.** 2001. Enhanced cellular immune response and reduced CD8(+) lymphocyte apoptosis in acutely SIV-infected Rhesus macaques after short-term antiretroviral treatment. Virology **279:**221-32.

274. **St Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557-9.

275. **Stahl-Hennig, C., R. M. Steinman, K. Tenner-Racz, M. Pope, N. Stolte, K. Matz-Rensing, G. Grobschupff, B. Raschdorff, G. Hunsmann, and P. Racz.** 1999. Rapid infection of oral mucosal-associated lymphoid tissue with simian immunodeficiency virus. Science **285:**1261-5.

276. **Staprans, S. I., P. J. Dailey, A. Rosenthal, C. Horton, R. M. Grant, N. Lerche, and M. B. Feinberg.** 1999. Simian immunodeficiency virus disease course is predicted by the extent of virus replication during primary infection. J Virol **73:**4829-39.

277. **Stevenson, M.** 2005. Developments in basic science research. Top HIV Med **13:**4-8.

278. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. Embo J **9:**1551-60.

279. **Strack, B., A. Calistri, S. Craig, E. Popova, and H. G. Gottlinger.** 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell **114:**689-99.

280. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature **427:**848-53.

281. **Taber, R., P. A. Rajakumar, D. H. Fuller, A. M. Trichel, P. Dowling, D. Meleason, A. Amedee, and M. Murphey-Corb.** 2006. Effects of monotherapy with (R)-9-(2-phosphonylmethoxypropyl)adenine (PMPA) on the evolution of a primary Simian immunodeficiency virus (SIV) isolate. Reprinted from Virology **354:**116-31, with permission from Elsevier.

282. **Tang, J., S. Tang, E. Lobashevsky, A. D. Myracle, U. Fideli, G. Aldrovandi, S. Allen, R. Musonda, and R. A. Kaslow.** 2002. Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1. J Virol **76:**8276-84.

283. **Tang, J. W., and D. Pillay.** 2004. Transmission of HIV-1 drug resistance. J Clin Virol **30:**1-10.

284. **Thompson.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:**4876-82.

285. **Tremblay, M., and M. A. Wainberg.** 1990. Neutralization of multiple HIV-1 isolates from a single subject by autologous sequential sera. J Infect Dis **162:**735-7.

286. **Trichel, A. M., P. A. Rajakumar, and M. Murphey-Corb.** 2002. Species-specific variation in SIV disease progression between Chinese and Indian subspecies of rhesus macaque. J Med Primatol **31:**171-8.

287. **Trichel, A. M., E. D. Roberts, L. A. Wilson, L. N. Martin, R. M. Ruprecht, and M. Murphey-Corb.** 1997. SIV/DeltaB670 transmission across oral, colonic, and vaginal mucosae in the macaque. J Med Primatol **26:**3-10.

288. **Trinchieri, G.** 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol **3:**133-46.

289. **Tryniszewska, E., J. Nacsa, M. G. Lewis, P. Silvera, D. Montefiori, D. Venzon, Z. Hel, R. W. Parks, M. Moniuszko, J. Tartaglia, K. A. Smith, and G. Franchini.** 2002. Vaccination of macaques with long-standing SIVmac251 infection lowers the viral set point after cessation of antiretroviral therapy. J Immunol **169:**5347-57.

290. **Tsai, C. C., K. E. Follis, A. Sabo, T. W. Beck, R. F. Grant, N. Bischofberger, R. E. Benveniste, and R. Black.** 1995. Prevention of SIV infection in macaques by (R)-9-(2-phosphonylmethoxypropyl)adenine. Science **270:**1197-9.

291. **Turner, D., and M. A. Wainberg.** 2006. HIV transmission and primary drug resistance. AIDS Rev **8:**17-23.

292. **Ulmer, J. B., R. R. Deck, C. M. DeWitt, A. Friedman, J. J. Donnelly, and M. A. Liu.** 1994. Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. Vaccine **12:**1541-4.

293. **UNAIDS** 2006 Global Report. http://www.unaids.org/en/HIV_data/2006GlobalReport/default.asp

294. **UNAIDS** 2004, Epidemic Update. http://www.unaids.org/wad2004/EPIupdate2004_html_en/epi04_00_en.htm

295. **UNAIDS**, 2006, Financing the response to AIDS. http://www.unaids.org/en/HIV_data/2006GlobalReport/default.asp

296. **Unutmaz, D., V. N. KewalRamani, and D. R. Littman.** 1998. G protein-coupled receptors in HIV and SIV entry: new perspectives on lentivirus-host interactions and on the utility of animal models. Semin Immunol **10:**225-36.

297. **Vajdy, M., R. Veazey, I. Tham, C. deBakker, S. Westmoreland, M. Neutra, and A. Lackner.** 2001. Early immunologic events in mucosal and systemic lymphoid tissues after intrarectal inoculation with simian immunodeficiency virus. J Infect Dis **184:**1007-14.

298. **Van Rompay, K. K., J. M. Cherrington, M. L. Marthas, C. J. Berardi, A. S. Mulato, A. Spinner, R. P. Tarara, D. R. Canfield, S. Telm, N. Bischofberger, and N. C. Pedersen.** 1996. 9-[2-(Phosphonomethoxy)propyl]adenine therapy of established simian immunodeficiency virus infection in infant rhesus macaques. Antimicrob Agents Chemother **40:**2586-91.

299. **Van Rompay, K. K., M. D. Miller, M. L. Marthas, N. A. Margot, P. J. Dailey, D. R. Canfield, R. P. Tarara, J. M. Cherrington, N. L. Aguirre, N. Bischofberger, and N. C. Pedersen.** 2000. Prophylactic and therapeutic benefits of short-term 9-[2-(R)-(phosphonomethoxy)propyl]adenine (PMPA) administration to newborn macaques following oral inoculation with simian immunodeficiency virus with reduced susceptibility to PMPA. J Virol **74:**1767-74.

300. **Van Rompay, K. K., R. P. Singh, L. L. Brignolo, J. R. Lawson, K. A. Schmidt, B. Pahar, D. R. Canfield, R. P. Tarara, D. L. Sodora, N. Bischofberger, and M. L. Marthas.** 2004. The clinical benefits of tenofovir for simian immunodeficiency virus-infected macaques are larger than predicted by its

effects on standard viral and immunologic parameters. J Acquir Immune Defic Syndr **36:**900-14.

301. **Van Rompay, K. K., R. P. Singh, B. Pahar, D. L. Sodora, C. Wingfield, J. R. Lawson, M. L. Marthas, and N. Bischofberger.** 2004. CD8+-cell-mediated suppression of virulent simian immunodeficiency virus during tenofovir treatment. J Virol **78:**5324-37.

302. **Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner.** 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280:**427-31.

303. **Veazey, R. S., I. C. Tham, K. G. Mansfield, M. DeMaria, A. E. Forand, D. E. Shvetz, L. V. Chalifoux, P. K. Sehgal, and A. A. Lackner.** 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. J Virol **74:**57-64.

304. **Veenstra, J., I. G. Williams, R. Colebunders, L. Dorrell, S. E. Tchamouroff, G. Patou, J. M. Lange, I. V. Weller, J. Goeman, S. Uthayakumar, I. R. Gow, J. N. Weber, and R. A. Coutinho.** 1996. Immunization with recombinant p17/p24:Ty virus-like particles in human immunodeficiency virus-infected persons. J Infect Dis **174:**862-6.

305. **Villani, N., R. Calio, E. Balestra, J. Balzarini, E. De Clercq, E. Fabrizi, C. F. Perno, and V. Del Gobbo.** 1994. 9-(2-Phosphonylmethoxyethyl) adenine

increases the survival of influenza virus-infected mice by an enhancement of the immune system. Antiviral Res **25:**81-9.

306. **Voronin, Y., J. Overbaugh, and M. Emerman.** 2005. Simian immunodeficiency virus variants that differ in pathogenicity differ in fitness under rapid cell turnover conditions. J Virol **79:**15091-8.

307. **Wagner, L., O. O. Yang, E. A. Garcia-Zepeda, Y. Ge, S. A. Kalams, B. D. Walker, M. S. Pasternack, and A. D. Luster.** 1998. Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans. Nature **391:**908-11.

308. **Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell.** 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med **333:**1038-44.

309. **Watson, A., J. Ranchalis, B. Travis, J. McClure, W. Sutton, P. R. Johnson, S. L. Hu, and N. L. Haigwood.** 1997. Plasma viremia in macaques infected with simian immunodeficiency virus: plasma viral load early in infection predicts survival. J Virol **71:**284-90.

310. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307-12.

311. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, and et al.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117-22.

312. **Wilson, L. A., M. Murphey-Corb, L. N. Martin, R. M. Harrison, M. S. Ratterree, and R. P. Bohm.** 2000. Identification of SIV env-specific CTL in the jejunal mucosa in vaginally exposed, seronegative rhesus macaques (Macaca mulatta). J Med Primatol **29:**173-81.

313. **Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman.** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278:**1291-5.

314. **Yang, N. S., J. Burkholder, B. Roberts, B. Martinell, and D. McCabe.** 1990. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci U S A **87:**9568-72.

315. **Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker, and R. P. Johnson.** 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. J Virol **70:**5799-806.

316. **Yasutomi, Y., S. Koenig, S. S. Haun, C. K. Stover, R. K. Jackson, P. Conard, A. J. Conley, E. A. Emini, T. R. Fuerst, and N. L. Letvin.** 1993. Immunization with recombinant BCG-SIV elicits SIV-specific cytotoxic T lymphocytes in rhesus monkeys. J Immunol **150:**3101-7.

317. **Yasutomi, Y., K. A. Reimann, C. I. Lord, M. D. Miller, and N. L. Letvin.** 1993. Simian immunodeficiency virus-specific CD8+ lymphocyte response in acutely infected rhesus monkeys. J Virol **67:**1707-11.

318. **York, I. A., and K. L. Rock.** 1996. Antigen processing and presentation by the class I major histocompatibility complex. Annu Rev Immunol **14:**369-96.

319. **Yoshimura, K., E. Ido, H. Akiyama, T. Kimura, M. Aoki, H. Suzuki, H. Mitsuya, M. Hayami, and S. Matsushita.** 2003. The impact of highly active antiretroviral therapy by the oral route on the CD8 subset in monkeys infected chronically with SHIV 89.6P. J Virol Methods **112:**121-8.

320. **Zack, J. A., A. M. Haislip, P. Krogstad, and I. S. Chen.** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol **66:**1717-25.

321. **Zanussi, S., C. Simonelli, M. D'Andrea, C. Caffau, M. Clerici, U. Tirelli, and P. DePaoli.** 1996. CD8+ lymphocyte phenotype and cytokine production in long-term non-progressor and in progressor patients with HIV-1 infection. Clin Exp Immunol **105:**220-4.

322. **Zhang, D., A. M. Caliendo, J. J. Eron, K. M. DeVore, J. C. Kaplan, M. S. Hirsch, and R. T. D'Aquila.** 1994. Resistance to 2',3'-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother **38:**282-7.

323. **Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao.** 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature **424:**94-8.

324. **Zhang, J. Y., L. N. Martin, E. A. Watson, R. C. Montelaro, M. West, L. Epstein, and M. Murphey-Corb.** 1988. Simian immunodeficiency virus/delta-induced immunodeficiency disease in rhesus monkeys: relation of antibody response and antigenemia. J Infect Dis **158:**1277-86.

325. **Zhang, L., C. Chung, B. S. Hu, T. He, Y. Guo, A. J. Kim, E. Skulsky, X. Jin, A. Hurley, B. Ramratnam, M. Markowitz, and D. D. Ho.** 2000. Genetic characterization of rebounding HIV-1 after cessation of highly active antiretroviral therapy. J Clin Invest **106:**839-45.

326. **Zhang, L., P. J. Dailey, T. He, A. Gettie, S. Bonhoeffer, A. S. Perelson, and D. D. Ho.** 1999. Rapid clearance of simian immunodeficiency virus particles from plasma of rhesus macaques. J Virol **73:**855-60.

327. **Zhang, L., T. He, Y. Huang, Z. Chen, Y. Guo, S. Wu, K. J. Kunstman, R. C. Brown, J. P. Phair, A. U. Neumann, D. D. Ho, and S. M. Wolinsky.** 1998. Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. J Virol **72:**9307-12.

328. **Zhu, T., D. Muthui, S. Holte, D. Nickle, F. Feng, S. Brodie, Y. Hwangbo, J. I. Mullins, and L. Corey.** 2002. Evidence for human immunodeficiency virus type 1 replication in vivo in CD14(+) monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. J Virol **76:**707-16.

329. **Zhu, T., N. Wang, A. Carr, D. S. Nam, R. Moor-Jankowski, D. A. Cooper, and D. D. Ho.** 1996. Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission. J Virol **70:**3098-107.

330. **Zhuang, J., A. E. Jetzt, G. Sun, H. Yu, G. Klarmann, Y. Ron, B. D. Preston, and J. P. Dougherty.** 2002. Human immunodeficiency virus type 1 recombination: rate, fidelity, and putative hot spots. J Virol **76:**11273-82.

331. **Zidek, Z., D. Frankova, and A. Holy.** 2001. Activation by 9-(R)-[2-(phosphonomethoxy)propyl]adenine of chemokine (RANTES, macrophage inflammatory protein 1alpha) and cytokine (tumor necrosis factor alpha, interleukin-10 [IL-10], IL-1beta) production. Antimicrob Agents Chemother **45:**3381-6.