CLEAVAGE SITE COMPENSATORY SUBSTITUTIONS PARTIALLY RESTORE FITNESS TO SIMIAN IMMUNODEFICIENCY VIRUS VARIANTS

by

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Cleave Site Compensatory Substitutions Partially Restore Fitness to Simian Immunodeficiency Virus Variants

Oscar B. Alcoreza Jr.

Abstract

The human immunodeficiency virus is presently one of the most significant global health issues to date, with a disease burden that encumbers developing and developed nations alike. Although current antiretroviral therapy can help patients maintain undetectable levels of the virus throughout their bodies, once the treatment is ceased, the virus will rebound and disease progression continues. Thus, modalities to: 1-stop HIV transmission and spread, or 2- eradicate the virus once it is acquired are both urgently needed.

In this project, we seek to evaluate and understand the impact of a candidate vaccine therapy that targets the HIV protease cleavage sites (PCS) on viral fitness. Vaccination with this modality in a monkey model induces mutations at virus regions that are intolerant to change, presumably affecting the “fitness” of viral strains recovered from vaccines. Preliminary results of the study show that in the vaccine group (n=11), a disruption to one or more of the HIV protease cleavage sites results in improved maintenance of CD4+ T cells compared to unvaccinated controls (n=5). Furthermore, a correlation between the percentage of PCS mutations and reductions in viral load were seen. Our data indicate that the most common sites of mutation occur at two cleavage regions PCS2 and PCS12. We used site directed mutagenesis to introduce multiple PCS mutations into infectious clones of SIV. Our ongoing studies are evaluating the viral
fitness of the SIV mutants in a cell lines and PBMC using competitive viral fitness assays. The data from these studies will help inform in the areas of vaccine and therapy development for HIV-1.
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<td>AIDS</td>
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<td>Ad5</td>
<td>adenovirus serotype 5</td>
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<td>antiretroviral therapy</td>
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<td>Bp</td>
<td>basepair</td>
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<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
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<td>CD4</td>
<td>cluster of differentiation 4 glycoprotein</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>cytotoxic T lymphocyte</td>
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<td>C-X-C chemokine receptor type 4</td>
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<td>DNA</td>
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<td>Env</td>
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<td>Gag</td>
<td>group specific antigen</td>
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<td>p27</td>
<td>27 KD SIV capsid antigen</td>
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<tr>
<td>gp</td>
<td>glycoprotein</td>
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<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<td>HIV-1/HIV-2</td>
<td>human immunodeficiency virus type 1/ type 2</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen (several)</td>
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<td>IFN</td>
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<td>Ig</td>
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<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<td>LTNP</td>
<td>long-term non-progressor</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MOI</td>
<td>multiplicities of infection</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>Nab</td>
<td>neutralizing antibody</td>
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<td>Nef</td>
<td>negative regulatory factor</td>
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<td>nt</td>
<td>nucleotide</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>primer binding site</td>
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<td>protease cleavage sites</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PHA</td>
<td>phytohemagglutinin</td>
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<tr>
<td>Pol</td>
<td>polymerase</td>
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<td>Rgp120</td>
<td>recombinant glycoprotein120</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase- polymerase chain reaction</td>
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<tr>
<td>SHIV</td>
<td>simian-human immunodeficiency virus</td>
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<tr>
<td>SIVmac251</td>
<td>simian immunodeficiency virus 251 (biological quasispecies)</td>
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<tr>
<td>SIVmac239</td>
<td>simian immunodeficiency virus 239 (clone derived from SIV251)</td>
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VSV  vesicular stomatitis virus
INTRODUCTION

HIV and AIDS:

In 1984 several laboratories independently isolated viruses thought to be the etiologic agent responsible for acquired immune deficiency syndrome (AIDS) (Levy, Jay, 2007). These virus isolates were determined to be the same agent and was ultimately given the name human immunodeficiency virus (HIV). Since the discovery that HIV was responsible for AIDS, two species of the virus have been isolated, HIV-1 and HIV-2. HIV-1 is the most prominent and infectious strain of HIV (Levy, Jay, 2007). HIV exhibits broad diversity with four HIV-1 sub-groups and eight HIV-2 groups having been characterized. HIV-1 group M (Major) is responsible for the current AIDS epidemic and nine clades have also been identified. HIV infection has three general clinical stages that include acute infection, clinical latency (lasting an average of 3-10 years) and AIDS (characterized by a CD4+ cell count of 200cells/mm3 or lower). Individuals left untreated with AIDS survive an average of 3 years (AIDS.gov).

Transmission of HIV-1 occurs principally through the exchange of genital secretions, blood or breast milk that contain cell-associated and cell-free virus. Sexual transmission is the most common route of transmission, yet is the least efficient with 1 out of 1000 sexual encounters resulting in a transmission event (Levy, Jay, 2007). Sexual transmission can be increased by several factors including circumcision status, presence of inflammation or ulcers in genital mucosa. Although, transmission from mother-to-child via breast feeding and the sharing of needles among intravenous drug users are
higher risk transmission routes, they have low overall incidence rates and contribute substantially less to HIV prevalence (Sagar, 2014).

In 2011, there were an estimated 34.2 million persons living with HIV, of which 2.5 million were new infections and 1.7 million died (Lou, Chen, Yu, Li, & Ye, 2014). Despite these sobering statistics, there has been significant progress in HIV treatment in the past few decades. The advent of antiretroviral therapy (ART) has been the greatest success in HIV/AIDS medicine to date (Richman et al., 2009). The life expectancy of a person living in the US with HIV starting ART at 20 years old has increased by 15 years from 2000 to 2006 (Samji et al., 2013).

In the US, HIV-1 infection is now considered a chronic, manageable disease. Unfortunately the majority of people living with HIV-1 live in low-income countries where life-long ART is not readily available, nor a feasible treatment option (Dorling, Shaw, & Davey Smith, 2006). These facts raise an impetus to go beyond managing life-long HIV/AIDS, which comes at a health and financial cost, to developing an efficacious prophylactic vaccine and potentially a cure for already-infected patients.

**HIV Genetic Organization**

HIV belongs to the genus *Lentivirus* within the family *Retroviridae*. The HIV virion is composed of a conical protein capsid housing two copies of the positive single-stranded RNA genome and the enzymes reverse transcriptase, integrase and protease (Kartikeyan, Bharmal, Tiwari, & Bisen, 2007). Surrounding the viral capsid is a two
layered envelope that is derived from the host cell membrane as progeny virus leave the host in a process termed “virus budding” (Sundquist & Krausslich, 2012). The outermost viral envelope contains glycoprotein subunits that project out of the envelope itself, forming a dense “glycan or sugar shield” that aids immune escape and evasion by blocking host antibody recognition of “glycan masked” envelope proteins. Glycoprotein 120 (gp120) is one such projection that is connected to the virion via a transmembrane protein, termed glycoprotein 41 (gp41). Gp120 is responsible for binding to target cells which express the CD4 receptor (Melikyan, 2014).

The HIV-1 genome can be grouped by genes encoding non-structural genes (*tat, rev, nef, vpr, vif* and *vpu*), which regulate the replication cycle and differentiate HIV from other retroviruses, and structural proteins (*gag, env* and *pol*), which are common to all retroviruses (Kartikeyan *et al.*, 2007). The *tat* gene expresses Tat (trans-activating) protein that activates the transcription of all viral genes. The *rev* gene produces Rev (regulatory of virus) protein activates expression of both structural and enzymatic genes. The *nef* gene produces Nef (negative factor) protein that is believed to regulate viral latency. The *vif* gene expresses Vif (viral infectivity factor) protein that assists the viral budding process. The *vpr* gene expresses Vpr protein and has been shown to stimulate the promoter region of HIV. The *vpu* gene, present only in HIV-1, encodes for various small proteins whose functions remain unknown, but is believed to play a role in mediating the release of budding new progeny virus (Dubé, Bego, Paquay, & Cohen, 2010).

The *gag* gene, is the major HIV structural component and encodes the (3) virion core and capsid proteins (CA, NC and p2). The *env* gene encodes the envelope
glycoproteins gp120, responsible for CD4 binding, and gp41, which is involved in the fusion of viral and host cell membranes (Sundquist & Krausslich, 2012). The pol gene encodes the enzymes reverse transcriptase, integrase and protease. Reverse transcriptase converts the single stranded RNA genome into a DNA duplex once inside the host cell. Integrase is responsible for integrating the DNA duplex into the host cell genome as proviral DNA. Protease is the enzyme that cleaves all the precursor polypeptides into functional proteins and is essential for the formation of infectious virus (Pokorná, Machala, Úzová, & Konvalinka, 2009).

**HIV Protease**

The HIV-1 Protease is biologically active as a symmetrical dimer and is the target of 9 Protease Inhibitors (Saquinavir, Indinavir, Ritonavir, Nelfinavir, Lopinavir, Atazanavir, Fosamprinavir, Tiranavir and Darunavir) used in ART (Warnke, Barreto, & Temesgen, 2007). The protease enzyme is active during the later phase in the HIV replication cycle when precursor polypeptides transcribed from proviral DNA are cut at 12 conserved protease cleavage sites (PCS), thus activating the individual proteins/enzymes into their functional forms (de Oliveira et al., 2003; Kartikeyan et al., 2007).
**HIV-1 Reverse Transcriptase**

The HIV Reverse Transcriptase (RT) is a critical component of the virus and unique in its ability to reverse transcribe RNA to DNA. The recognition of this ability resulted in the award of a Nobel Prize (Temins, Baltimore and Dulbecco). HIV-1 RT lacks a proofreading mechanism which results in $3 \times 10^{-5}$ mutations per base pair per cycle. This high mutation rate coupled with a fast replication cycle of $10^{10}$ viral particles per day enables HIV to exist in a host as quasi-species with subtle mutations that persist in response/adaptation to host immune pressure (Coffin, 1995; Tebit, Nankya, Arts, & Gao, 2007). A 1995 study by Ho et al., revealed that the half-life of HIV in vivo was around 2 days, representing an extremely rapid turnover rate (Ho et al., 1995). These properties enable HIV to respond to the host immune pressure, and any potential therapy, through the natural selection of the “most-fit” virus in which any beneficial mutation is rapidly selected for and maintained. The HIV-1 RT was initially the primary target for antiretroviral therapy and an entire class of molecules termed “RT inhibitors” (including 3TC and AZT, PMPA and FTC) underscore the level of work in this area.

The full HIV genome as well as the mature HIV virion are shown in Figure 1.
**Figure 1.** (A) Illustration of the full HIV genome structure including the long-terminal repeats (LTR) (B) Illustration of the HIV virion including the major membrane, capsid and matrix proteins as well as key enzymes. Figure adapted from Shum *et al.*, 2013.

**Viral Evolution in Response to Host Immunity**

HIV preferentially infects CD4+ T-cells, using the CD4 receptor and CXCR4, CCR5 co-receptors to gain cellular entry (Mogensen, Melchjorsen, Larsen, & Paludan, 2010). There is also evidence that HIV-1 trans infection of CD4+ T-cells by professional antigen presenting cells (APCs), such as macrophages, dendritic cells and B-lymphocytes, could be a major contributor to the precipitous decline of CD4+ T-cells
seen as the infection progresses (Rinaldo, 2013). As CD4+ T-cells play an important role in adaptive immunity, there is a strong negative correlation between declining CD4+ T-cell counts and progression to AIDS (Paiardini & Müller-Trutwin, 2013). CD4+ Central Memory T-cells represent the largest infected reservoir of CD4+ T-cells due to their role in maintaining normal CD4+ T-cell levels in the body, termed homeostasis (Paiardini & Müller-Trutwin, 2013).

Studies examining virus from individuals with acute HIV infection provided insight into the complex interactions between HIV and host immunity. In these patients neutralizing antibodies (Nab) were detected as early as 52 days after confirmation of infection. However, these Nab became ineffective after complete replacement of Nab sensitive virus with virus that contained mutations in HIV Env which conferred resistance to the Nab (Wei et al., 2003). This study also proposed the idea that HIV possesses a “glycan shield” capable of high diversity to prevent Nab binding without affecting receptor binding. In addition to neutralization escape, HIV is capable of acquiring drug resistance through the maintenance of mutations rendering antiretroviral drugs ineffective (Tang & Shafer, 2012). However, researchers have demonstrated that drug resistance mutations almost invariably come at a fitness cost to the virus. Thus, the drug-resistant virus must sacrifice replicative fitness to persist in the presence of the drug.

During acute infection, the initial decline in virus is believed to occur due to increases in CD8+ T-cells, or cytotoxic T-lymphocytes (CTL), which are critical for viral control and help in establishing the viral load set-point (Koup et al., 1994; McMichael, Borrow, Tomaras, Goonetilleke, & Haynes, 2010). This viral load set point represents the
stable production of virus in HIV+ individuals as the balance between HIV replication and host immune system pressure is reached.

Studies analyzing the immune systems of individuals capable of controlling HIV infection without ART, termed elite controllers (EC) or long-term non-progressors (LTNP), have found that there is a strong correlation between certain major histocompatibility complex (MHC) alleles and increased HIV-1 control (Zaunders & van Bockel, 2013). Similarly to escape from antibodies and antiretroviral drugs, HIV-1 is able to produce and maintain CTL escape mutations. Investigation into CTL escape mutations revealed that a small portion of these mutations come at a high fitness cost to the virus and often require compensating mutations at other sites on the virus genome, which mitigate the fitness cost of CTL escape (Goulder & Watkins, 2004). This “fitness cost” has been demonstrated through ex vivo studies of HIV with CTL escape mutations against HLA-B57 infecting a population of cells without HLA-B57. For example, the loss of immune pressure towards the maintenance of the B57 escape mutation caused rapid reversion of mutant HIV, with CTL-escape mutations, back to the more pathogenic wild-type sequences (Leslie et al., 2004).

**Ongoing Hurdles in HIV-1 Vaccine Development**

Despite over three decades of HIV research there has been only four HIV-1 vaccine candidates that have managed to reach clinical efficacy trials.

The first HIV vaccine candidate to reach clinical efficacy trials was one that used recombinant HIV-1 envelope glycoprotein subunit (rgp120) to attempt to induce a potent
immune response capable of eliciting broad, potent neutralizing antibodies (Group, 2005). The results of this study concluded that the vaccine provided no overall protective effects. The second vaccine candidate was an adenovirus serotype 5 (Ad5) vector vaccine which expressed viral Gag, Pol and Nef proteins. The primary objective of this trial was to reduce HIV-1 acquisition rates and viral set point in vaccine recipients primarily through cell-mediated immunity. Although an increase in CTL response was observed, via IFN-γ ELISPOT, the results of this study also showed no protective effects and may have increased the risk of infection, as the vaccine group had a higher incidence of infection than the control group (Buchbinder et al., 2008). The third vaccine candidate was a prime-boost vaccine regimen involving four priming injections of recombinant canarypox vaccine vector, followed by two booster injections of the rgp120 vaccine. This candidate vaccine was able to provide a modest efficacy of prevention of HIV incidence of 31.2%. However, the vaccine failed to modify the degree of viremia or CD4+ T-cell counts of those who became infected (Rerks-Ngarm et al., 2009). Additionally, the protective effect waned rapidly following treatment. The last vaccine candidate to reach clinical efficacy trials was a DNA prime-recombinant Ad5 boost regimen which was stopped early due to lack of efficacy (Hammer et al., 2013)(Barouch, 2013).

**Barriers to a Cure for HIV**

To date, there is but a single documented case of a “sterilizing” or absolute HIV-1 cure, in the well-described “Berlin Patient” (Hütter et al., 2009). This is likely due to the fact that HIV exhibits numerous characteristics that have defied the development of a
curative treatment. These include its preferential infection of immune cells, rapid turnover, high mutation rate, and viral latency.

Despite ART’s ability to control HIV infection long-term with minimal clinical symptoms, a persistent, replication competent HIV reservoir has been shown to exist in patients on ART (T. W. Chun et al., 1997; Finzi et al., 1999). Finzi et al., attempted to characterize the decay kinetics of the latently infected resting CD4+ T-cell reservoir, on potent ART, and estimated that eradication of the latent reservoir could take as long as 60 years (Finzi et al., 1999; Ho et al., 1995)

This replication competent latent reservoir represents the next major obstacle in achieving a sterilizing cure for HIV. Chun et al., demonstrated that in a patient who had been on ART for over a decade, had undetectable plasma proviral DNA (from latently infected cells) and undetectable proviral DNA in the gut associated lymphoid tissue (GALT), nevertheless had a rebound of plasma viremia 50 days after discontinuation of ART (T.-W. Chun et al., 2010). A study by Whitney et al., revealed that the use of ART for 24 weeks starting as early as three days post infection in rhesus monkeys was not sufficient in preventing the formation of a latent reservoir (Whitney et al., 2014). These studies demonstrate that a successful sterilizing therapy must be capable of eliminating all traces of replication competent HIV, given that a small, undetectable reservoir, as small as a single infected cell, is capable of reigniting the infection (Ananworanich & Robb, 2014; Persaud et al., 2013).
Generally, the concept behind HIV-1 immunotherapy (a vaccine or other therapeutic) is a therapy that is able to diminish the pathogenicity of HIV-1 and/or strengthen a patient’s immune system to the point where it is able to keep the virus in check (Blankson, Siliciano, & Siliciano, 2014). Many unique clinical cohorts exist that can inform the rationale design of vaccines or similar immune therapies for HIV-1. This concept for a “functional cure” is based on the study of ECs and LTNPs, who are individuals infected with or exposed to HIV but do not progress to AIDS due to an immune system capable of effectively controlling the infection. Through the study of ECs, researchers have learned how the body can naturally control infection and scientists can use these insights in the development of more effective treatments.

Human leukocyte antigens (HLA) are the human equivalent of the MHC gene family that is involved in immune system pathogen recognition. These host proteins play an important role in immune surveillance through the binding and presentation of self and foreign proteins to T-cells. There is an enormous diversity of HLA alleles that contribute to variable levels of peptide binding and recognition in the human population. Study of ECs has revealed a high percentage of specific HLA alleles that are thought to contribute to better viremic control. Specifically, HLA-B*2705, HLA-B*5701 and HLA-B*5703 alleles have been extensively studied due to their presence in EC (Zaunders & van Bockel, 2013) (Migueles et al., 2000).

Another unique set of individuals is the Pumwani Commercial Sex Worker
Cohort study involved the observation of 424 initially seronegative sex workers in Nairobi, Kenya from 1985-1994. The results of this study demonstrated that 43 women in this group remained seronegative throughout 3+ years of follow up. Despite an estimated 500 unprotected exposures to HIV-1, a small group of women appeared to have a natural immunity to HIV-1 (Fowke et al., 1996). One study investigating the CTL response in this cohort revealed that 40% of the HIV seronegative individuals had an IFN-gamma response, which corresponds to cytotoxic function, five times less than the HIV+ group. The seronegative group also had a 10-fold lower HIV-envelope specific CD8+ T-cell response, responding to only a single HIV peptide, compared to the HIV+ group which responded to an average of 5.5 peptides (Alimonti et al., 2006). Comparison of HLA allele A*01:01, which is represented in the Pumwani seronegative group, and HLA allele B*07:02, which is associated with susceptibility for HIV-1 infection, revealed that the B*07:02 allele bound ten times more HIV-1 Gag epitopes than the protective A*01:01 allele (M. Luo et al., 2012).

These observations reveal the potential requirement of an effective curative treatment to elicit a highly specific, narrow immune response, in contrast to the strong, broad immune response elicited by some of the previously described experimental vaccines.

In addition to natural immunity there have been cases of non-progression due to infection by attenuated viruses. A study examining the HIV-1 nef gene sequences of five LTNP revealed one patient who generated only defective forms of nef in 34 blood samples obtained in a 10 year period (Kirchhoff, Greenough, Brettler, Sullivan, &
Desrosiers, 1995). The disease progression of this patient was similar to the progression of rhesus monkeys inoculated with a SIVmac239 clone containing a defective nef gene. This case demonstrates the potential of a normal individual, infected predominantly by an attenuated form of HIV, being capable of controlling viremia and disease progression.

**Importance of Viral Fitness in Therapy**

Viral fitness is a multifaceted term that describes HIV’s replicative ability within a given environment. Due to the unique nature of HIV infection and its replicative characteristics, descriptions of fitness should be approached cautiously. However, given the observations that escape mutations often develop at the expense of replicative fitness, it may be possible to develop a therapeutics, that generate selective pressures strong enough to force HIV to mutate into an attenuated variant that an individual’s immune system can then control. Studies have shown that HIV-positive individuals who maintain viral loads lower than 1,700 copies per ml were not able to transmit HIV to their partners (Goulder & Watkins, 2004). This highlights the complexity of describing HIV-1 fitness vis-à-vis virus transmission, control of viremia and disease progression (see Figure 2).

Towards this end it is necessary to have an accurate mechanism to measure relative fitness. A study by Peyerl et al., assessed the fitness loss of a rare SHIV CTL escape mutation (T47I) at a highly conserved Gag epitope and an associated compensatory mutation (I71V) (Peyerl et al., 2003). The T47I mutation alone resulted in a substantial decrease in gag p27 expression however a mutant clone with both T47I and I71V mutations “reverted” and expressed p27 at near wild-type levels. Another
determinant of viral fitness examined in this study included viral infectivity and replication. To measure infectivity, the env gene of mutated SHIV clones were replaced with the E. Coli CAT gene and pseudotyped with an HIV-1 envelope glycoprotein to allow entry into rhesus monkey PBMCs. Once again, the mutant virus containing only the T47I mutation expressed only background levels of CAT expression while the variant with both T47I and I71V mutations had wild-type levels of CAT expression. While these methods provide a limited way to compare viral fitness they do not represent the highly complex in vivo competition that exist between HIV quasi-species.

Quiñones-Mateu et al., developed a dual infection/competition assay that is able to accurately measure relative virus production and ex vivo fitness. This study used four control HIV-1 strains (two non-syncytium inducing/CCR5 tropic strains and two syncytium inducing/CXCR4) to evaluate the relative fitness of HIV-1 isolates from a Belgian cohort study. The results revealed that HIV-1 isolates from LTNPs were outcompeted by the control strains and were significantly less fit than HIV-1 isolates from patients with rapid progression to AIDS (Quiñones-Mateu et al., 2000). The results also revealed a direct correlation between ex vivo viral fitness and viral load. These results demonstrate that LTNP, possibly due to more effective CTL function, may be capable of attenuating HIV-1 infection to halt the progression to AIDS.

This type of reliable, ex vivo competition assay is critical for the further study and understanding of the contradictions that have been observed concerning CTL-mediated immune pressure and virus escape from immune control. For example, one study mentioned showed that fitness reducing CTL-escape mutants are capable of returning to
wild-type fitness through compensatory mutations (Peyerl et al., 2003), while another study demonstrates that LTNP, presumably through more effective CTL function, are capable of applying enough immune pressure to force the progression of less-fit HIV (Quiñones-Mateu et al., 2000). In order to advance the intelligent design of immune-based therapeutics, perhaps imitating the effective immune responses of natural LTNP and EC, it is vital to include and analyze comparative fitness of the dominant HIV-1 quasispecies during experimental treatments.

**Background of Current Investigation**

The Pumwani Sexworker Cohort study revealed the existence of the protective HLA A*01:01 gag epitope allele, which corresponds to a 9-mer peptide covering the HIV-1 protein cleavage site 1, (termed PCS 1). A recent study (Ma Luo et al., 2013) of immunogenicity and world population coverage of a vaccine targeting all 12 HIV-1 protease cleavage sites or “PCS” revealed that these sequences are immunogenic and many HLA class I alleles have target epitopes at multiple PCS.

Since the HIV-1 12 PCS are highly conserved (in order to maintain viral replication), we hypothesize that any deviation from the original sequence, due to immune-mediated immune pressure, will impact viral fitness. Knowing that the protective effect that the HLA A*01:01 CTL response confers, an experimental vaccine targeting the 12 highly conserved PCS on SIV was developed and tested in a monkey model of HIV-1 acquisition. Using 17 Cynomolgus macaques (*Macaca fascicularis*) as
our model for HIV, we tested the effects of a vaccine consisting of a cocktail of 20-mer peptides overlapping the 12 PCS. After vaccination, the animals were experimentally infected with a close analog of HIV- its simian counterpart, SIV.

Analysis of SIV sequences from all monkeys during the periods when they experienced high (ranging from weeks 2-4 post-infection) and low (ranging from weeks 6-17 post-infection) viral loads led to the selection of five mutations that dominated the virus population and persisted throughout the study in the majority of the subjects. Three of these mutations were located on PCS2 and two were located on PCS12.

The purpose of this thesis work is to examine the fitness of mutant clones of SIVmac239 containing the selected predominant mutations from Cynomolgus macaques that received an experimental vaccine consisting of a modified vesicular stomatitis virus (VSV) vector system expressing peptides overlapping 12 highly conserved SIV protease cleavage sites. We hypothesize that the mutant SIVmac239 clones will exhibit an altered fitness compared to a wild-type SIVmac239, unexposed to selective pressures. The long-term objective of this work is to further characterize deficits in viral fitness as a result of PCS-based vaccine strategies and response to immune pressure. This data will inform the development of vaccine concepts capable of eliciting effective immune control and preventing further transmission.
METHODS

Animals, SIV Challenge and Vaccination Protocol

This study enrolled and monitored adult Cynomolgus macaques (*Macaca fascicularis*). All animals were genotyped for their MHC haplotypes using PCR and MHC-amplicon sequencing. The monkeys were housed under biosafety level-2+ conditions, and routinely monitored by physical exam and T cell subset analysis before and after SIV infection.

Specimen Collection and Processing

Peripheral blood was collected weekly. PBMC from collected blood were separated on Ficoll-Hypaque cushions following standard protocols, while blood plasma was immediately frozen and maintained at –80°C until use. All PBMC cultures were first purified by CD8-depletion using a commercial MACS magnetic bead depletion assay (Miltenyibiotec inc.) and then cryopreserved and frozen at -180°C for later use. After thawing, all PBMC were stimulated for 48hrs with Concanavalin A, prior to use in experiments. PBMCs were maintained in lymphocyte medium (RPMI 1640 with 10% FBS and 40 U/ml IL-2).

Viral RNA Extraction and qRT-PCR Detection

Viral RNA was routinely isolated from 140 µL of blood plasma using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer’s protocol. RNA recovered from spin columns was eluted to a final volume of 60 µL.
cDNA Synthesis

A 10µL volume of extracted RNA was reverse transcribed to single-stranded cDNA following the manufacturer’s instructions in the Superscript III protocol (Invitrogen). RNA, deoxynucleotides (0.5 mM each), and 0.24 µM primer OR9608 (5’ – CTCATCTGATACATTTACGGG – 3’) were incubated for 5 minutes at 65°C. The sample was chilled on ice for 1 minute, followed by a brief centrifugation. First strand cDNA synthesis was completed by adding 10 µL of 5x reaction buffer, 2.5 µL each of 0.1 M DTT, RNase Out, and Superscript III RT. The mixture was then incubated for 60 minutes at 50°C, 60 minutes at 55°C and 15 minutes at 70°C. Finally, 5 units of RNase H were added and the mixture was incubated for 20 minutes at 37°C.

QPCR assay

The SIV RNA standard was transcribed from the pSP72 vector containing the first 731 bp of the SIVmac239-Gag gene using the Megascript T7 kit (Ambion Inc., Austin, TX). RNA was isolated by phenol-chloroform purification followed by ethanol precipitation. All purified RNA preparations were quantified by optical density. RNA quality was determined using Agilent bioanalyzer RNA chip (Agilent Santa, Clara CA).

QRT-PCR was conducted in a 2-step process. First, RNA was reverse transcribed in parallel with a SIV-gag RNA standard using the gene-specific primer sGag-R 5’CACTAGGTGTCTCTGCACATCTGTTTTG-3’ under the following conditions: the 50 µL reactions contained 1X buffer (250 mM Tris-HCL pH 8.3, 375 mM KCl, 15 mM MgCl2), 0.25 µM primer, 0.5 mM dNTPs (Roche), 5 mM dTT, 500 U Superscript III RT
(Invitrogen, Carlsbad, CA), 100 U RnaseOUT (Invitrogen, Carlsbad, CA), and 10 µL of sample. RT conditions consisted of 1 hour at 50˚C, 1 hour at 55˚C and 15 minutes at 70˚C. All samples were then treated with RNase H (Stratagene, Cedar Creek, TX) for 20 minutes at 37˚C. All real-time PCR reactions used EZ RT PCR Core Reagents (Applied Biosystems, Foster City, CA) following the manufacturer’s suggested instructions under the following conditions: the 50µL reactions contained 1X buffer (250 mM Bicine, 575 mM potassium acetate, 0.05 mM EDTA, 300 nM Passive Reference 1, 40% (w/v) glycerol, pH 8.2, 3 mM each of dATP, dCTP, dGTP, .6 mM dUTP, 3 mM Mn (OAc)2, .5 U uracil N-glycosylase, 5 U rTth DNA Polymerase, .4 uM of each primer, and 10µL of sample template. PCR reagents were assembled at room temperature and spun briefly to eliminate air bubbles. Following 2 minutes at 50˚C, the polymerase was activated for 10 minutes at 95˚C, and then cycling proceeded at 15 seconds at 95˚C and 1 minute at 60˚C for fifty cycles. Primer sequences were adapted from those described by Cline et al (Nichole Cline, Bess, Piatak, & Lifson, 2005), forward primer s-Gag-F: 5’-GTCTGCGTCATCTGGTGATCATTCC-3’, reverse primer s-Gag-R: 5’-CACTAGGTGTCTCTGCACATCTGATTTTG-3’, and the probe s-Gag-P: 5’-CTTCCTCAGTGTTTCACTTTTCTCTCTTGCG-3’, linked to Fam and BHQ (Invitrogen, Carlsbad, CA). All reactions were carried out on a 7300 ABI Real-Time PCR system (Applied Biosystems) in triplicate according to the manufacturer’s protocols.

**Construction of PCS mutant SIV clones**

The first step in the construction of the mutated SIV-PCS12- m-tracking vector, SIV-PCS12- m, involved subcloning of the region located between restriction sites SphI
(position 6701) and XhoI (position 10535) from the full-length infectious clone of SIVmac239, derived from a biological isolate of SIVmac251 as described by Whitney et al, into pSP73 (Promega). The resulting plasmid was subjected to site-directed mutagenesis in order to insert 2 nonsynonymous mutations into the PCS12 region using the Stratagene Quickchange II kit, as specified by the manufacturer. The mutagenized SphI-XhoI fragment was then inserted into the parental clone to produce the PCS-Mutant clone (PCS12-M). Infectious virus stocks were prepared by transient transfection of 293T cells with SIVmac239 wild type or PCS12-M plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

**Virus stocks**

Viruses used in the fitness studies are biological isolates and chimeric virus derived from the vaccine and control arms of a primate-based HIV-candidate vaccine study (Letvin *et al.*, 2006). Stocks of each isolates were prepared and all infectious stocks were stored in aliquots at -80 °C. The titers (50% tissue culture infectious dose (TCID50)) of all stocks were determined in PBMC culture by standard methods and data was analyzed using the method described by Spearman and Karber.

**Growth-competition assays**

Growth-competition assays were performed in 12-well plates seeded with 2e 106 PHA-activated rhesus PBMCs in 2 ml total volume. The two viruses under evaluation were added to the target cells at individual multiplicities of infection (MOI) of 0.001 or 0.005, which are generally accepted to be low enough to prevent recombination. To limit
the natural variability of PBMC replication assays, each data point was derived from
duplicate cultures on the same plate and all experiments were performed twice. The
competition cultures described were maintained for 14 days. Supernatants were taken at
day 3, 5, 7, 9 and 12. PBMC were harvested from these supernatants, washed twice with
phosphate-buffered saline (PBS), and pelleted for DNA extraction using the QIAamp
DNA Blood Mini Kit, following the manufacturer's instructions. Virus production in
culture fluids was monitored using RT-PCR and SIV p27 antigen capture assay (Coulter

**Single Genome Amplification of SIV Envelope**

To ensure that positive amplifications were a result of a single cDNA, viral cDNA
was diluted in 96-well plates to yield fewer than 30% wells positive for amplification
(Palmer *et al.*, 2005). First-round PCR was carried out in a reaction mixture containing:
1x buffer (Platinum Taq HF Kit, Invitrogen), 0.2 mM dNTP mix, 2mM Mg2SO4, 0.2 μM
primer OF6207 (5’ – GGGTAGTGAGGTTCTGGAAG – 3’), 0.2 μM primer OR9608
(5’ – CTCATCTGATACATTTACGGG – 3’), 0.025 units of Platinum Taq High
Fidelity polymerase into a total volume of 20 μL. PCR mixtures were then loaded into
MicroAmp Optical 96-Well Reaction Plates (Applied Biosciences). PCR conditions were
programmed using the following parameters: 5 minutes at 94°C, 35 cycles of 15 seconds
at 94°C, 30 seconds at 52°C, 4 minutes and 15 seconds at 68°C, followed by a final
extension time of 10 minutes at 68°C. For the second-round PCR, 2 μL of first-round
PCR product was mixed with 1x buffer (Platinum Taq HF Kit, Invitrogen), 0.2 mM
dNTP mix, 2mM Mg2SO4, 0.3 μM primer IF6428 (5’ –
CGTGCTATAACACATGCTATTG – 3’), 0.3 µM primer IR9351 (5’ – CCCTACCAAGTCATCATCTTC – 3’), 0.025 units of Platinum Taq High Fidelity polymerase into a total volume of 20 µL. Second round PCR conditions were programmed using the following parameters: 5 minutes at 94°C, 45 cycles of 15 seconds at 94°C, 30 seconds at 51°C, 3 minutes and 30 seconds at 68°C, followed by a final extension time of 10 minutes at 68°C. Amplicons from cDNA dilutions resulting in less than 30% positive reactions were sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Eight sequencing primers spanning the length of the SIV envelope were used for complete envelope sequencing. For each monkey, between 15-30 SIV envelope sequences were analyzed per time point.

SIV Envelope Sequence Analysis

Raw sequencing data was analyzed using the GeneCodes Sequencher 4.8 DNA sequencing software. Using Sequencher’s assembly algorithm groups of primer sequence fragments were assembled into overlapping contigs. Chromatograms of assembled contigs were manually corrected for each individual ambiguous base.

Alignments and Phylogenetic Analysis

After editing contig consensus sequences they were then exported for translation by the Los Alamos National Laboratory (LANL) HIV sequence database translate tool (http://www.hiv.lanl.gov) for further bioinformatic analysis. Nucleotide and translated contig consensus sequences were aligned with the online database EBI Tools ClustalW (Larkin et al., 2007) under default conditions. Nucleotide alignments were manually
aligned, when necessary, with the BioEdit Sequence Alignment Editor program in preparation for phylogenetic analysis.

Edited sequences were also sent to Dr. Bette Korber of the Los Alamos National Laboratory for phylogenetic analysis, a brief methodology is described below. All phylogenetic analysis of nucleotide sequences was performed using the MEGA 4 evolutionary analysis software package. Evolutionary trees were inferred using the maximum parsimony and neighbor-joining methods. The bootstrap consensus trees were inferred from 500 replicates and were taken to represent the evolutionary history of all taxa analyzed. Maximum parsimony trees were obtained using the Close-Neighbor-Interchange method in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the datasets.
RESULTS

_Vaccine targeting protease cleavage sites elicited control of viremia._

As classical vaccine approaches to elicit either T cell or antibody responses against HIV-1 has failed so far or produced a modest effect, new approaches are needed. The immunogenicity of the sequences around the protease cleavage sites (PCS) and the population coverage for a vaccine targeting HIV-1 PCS has been investigated (Ma Luo _et al._, 2013). In the pilot study, sequences around PCS were assessed as targets for the development of a new vaccine strategy using Philippine Cynomolgus macaques and SIVmac239 as an experimental model. The vaccine regimen included immunogens that are cocktails of 20mer peptides overlapping the 12-protease cleavage sites (PCS).

Both plasma SIV RNA levels and CD4+ T cell counts were monitored in this cohort of cynomolgus monkeys through 20 weeks after SIVmac239 challenge. No significant changes in viral load were observed within the vaccine or control group mostly due to higher dose of SIVmac239 used to infect the vaccine group (Figure 2A).
Figure 2. The kinetics of plasma SIV RNA and total CD4+ T cell counts following SIVmac239 infection. (A) Plasma virus RNA levels were assessed in cynomolgus monkeys between wks 1 to 20 after challenging with SIVmac239. Log₁₀ SIV RNA copies/mL for each monkey in both the vaccine (red) and control (blue) groups is shown. Median viral loads in both the vaccine (black solid) and control (black dashed) groups are also shown. (B) Changes in CD4+ T cell counts for each monkey in both the vaccine (red) and control (blue) groups over time were plotted.
However, the vaccine group maintained CD4+ T cell counts better than the control animals, despite having increased levels of virus challenge (Figure 2B, Table 1).

<table>
<thead>
<tr>
<th>Weeks after infection</th>
<th>Vaccine (n=11)</th>
<th>Control (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of animals with CD4 decline (%)</td>
<td># of animals with CD4 maintain (%)</td>
</tr>
<tr>
<td>MID11</td>
<td>5 (45)</td>
<td>6 (56)</td>
</tr>
<tr>
<td>MID13</td>
<td>4 (36)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>MID14</td>
<td>7 (64)</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Endpoint</td>
<td>5 (45)</td>
<td>6 (56)</td>
</tr>
</tbody>
</table>

**Table 1.** Change in total CD4+ T cell counts in cynomolgus macaques following infection. Monkeys in the cohort are shown in 2 groups, one of animals that showed decline in total CD4+ T cell counts and the other of animals that maintain CD4+ T cells.

This vaccine modality can generate antibody and T cell responses in macaques. Indeed, the antibody response to the peptides overlapping the PCS has been correlated with a reduction in viral load during the acute phase of SIV infection, while long-term durable control during the chronic stages, i.e. set-point is less pronounced (Figure 3A, B). Vaccine-elicited control of viral load was maintained over the course of 20 wks post-challenge as shown by significant associations between the vaccine elicited antibody response and an area-under-the curve calculation for the plasma SIV RNA levels between wk 1 and 20 following infection (Figure 3C, Table 2).
Figure 3. Correlation between vaccine-induced immune responses and plasma viral load in the vaccine group. Correlation matrices were generated using Spearman nonparametric correlation to compute the correlation coefficient for each pair of variables including plasma antibody and Elispot responses to number of PCS peptides and plasma SIV RNA levels in the vaccine group. The plasma SIV RNA levels were assessed on 2-4 weeks and 9-14 weeks following SIV challenge, representing peak (A) and viral set point (B) viral load, respectively. Area-under-the-curve calculations (C) for the plasma SIV RNA levels were also assessed in these monkeys.
Table 2. Correlation between vaccine-induced immune responses and plasma viral load in the vaccine group. The Spearman correlation coefficients ($\rho$) are shown. Exact P values for nonparametric Spearman correlation are shown.

Amino acid mutations in sequences around the PCS sites and correlations with viral load

As shown in Figure 4, twelve protease cleavage sites are located across the HIV-1 genome. Five cleavage sites are found on the Pr55Gag protein, six cleavage sites are found on the Gag-Pol protein, and one cleavage site is found on the Nef protein. Viral sequence analysis revealed extensive mutations around PCS regions (Figure 5) in both vaccinated monkeys and control monkeys over 20 wks after infection.
Figure 4. A schematic illustrating landmarks of SIVmac239 genome and 12 proteolytic cleavage sites. Twelve unique cleavage sites between Gag, Gag-Pol and Nef precursor polyproteins are designated.

Figure 5. The major PCS amino acid mutations of plasma SIVmac239 in a cohort of monkeys. The frequencies of each PCS amino acid mutation following infection with
SIVmac239 in the vaccine (A) and control (B) groups are shown in various colored lines and corresponding plasma SIV RNA copies are shown in black solid line.

Comparisons of the PCS mutation frequencies were conducted at each time point between the vaccine and control groups. The virus recovered from several vaccinated animals showed increased mutation frequencies in either PCS2 or PCS12 region. Specifically, virus recovered from the vaccine group showed significantly higher frequencies of mutation in PCS12 region ($P=0.009$) compared to the mutation frequencies in the control group (Figure 6). Amino acid mutation in PCS2 (-8) was associated with moderate yet significant increase of viral replication. However, amino acid mutation in PCS12 (-8) displayed a negative correlation with the plasma viral load (see Figure 7 below).
Figure 6. Comparison of the major PCS amino acid mutation frequencies between the vaccine and control groups. Total sum of frequencies found in the major PCS were compared. The comparison of the values from the groups of animals was determined using a non-parametric one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparison test or the Mann-Whitney test. The amino acid mutations less than 10% in other PCS sites are not included in the analysis.
Figure 7. Correlation between the major PCS amino acid mutation and changes in plasma viral load in the vaccine group. Correlation matrices were generated using Spearman nonparametric correlation to compute the correlation coefficient for each pair of variables including PCS amino acid mutations (PCS2: -8, -7, and -6 and PCS12: -8) and plasma SIV RNA levels in the vaccine group.

Construction of major PCS amino acid mutation.

In order to assess the effects of the PCS vaccine on SIV fitness and evolution, SIV was routinely isolated from blood samples drawn from Cynomolgus macaques used in the study. Analysis of the isolated SIV sequences from the monkeys during the periods when they experienced peak (ranging from weeks 2-4 post-infection) and set point (ranging from weeks 6-17 post-infection) viral loads led to the selection of 5 mutations which
persisted throughout the study in the majority of the subjects. Three of these mutations (PCS2-8, PCS2-7 and PCS2-6) occurred on PCS2, which lies on the sequence for the Gag capsid protein, p27, while the two other mutations (PCS12-8(E/R)) occurred on PCS12, which is on the sequence for the Nef regulatory protein. To introduce point mutations into each PCS region, the fragment between the BamHI and SphI sites, and the SphI and XhoI was subcloned into the pSP73 vector to generate a clone termed pSP73-PCS2, and the fragment between the SphI and XhoI sites was subcloned into the pSP73 vector to generate the clone termed pSP73-PCS12 (Figure 8A). The complete list of mutations inserted into the full length SIVmac239 genome is shown in Table 3.

<table>
<thead>
<tr>
<th>SIVmac239</th>
<th>PCS2</th>
<th>PCS12</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVmac239-WT</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-8)</td>
<td>R</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-7)</td>
<td>P</td>
<td>D</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-6)</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS12(-8R)</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS12(-8E)</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-8)/PCS12(-8R)</td>
<td>R</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-7)/PCS12(-8R)</td>
<td>P</td>
<td>D</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-6)/PCS12(-8R)</td>
<td>P</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-8)/PCS12(-8E)</td>
<td>R</td>
<td>G</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-7)/PCS12(-8E)</td>
<td>P</td>
<td>D</td>
</tr>
<tr>
<td>SIVmac239-PCS2(-6)/PCS12(-8E)</td>
<td>P</td>
<td>G</td>
</tr>
</tbody>
</table>

**Table 3.** Positional mutations induced by site-directed mutagenesis at each respective PCS site. Amino acid (AA) substitutions for each of the PCS mutations that were constructed are shown in red.
The QuikChange site-directed mutagensis kit (Stratagene, La Jolla, Calif.) was used to introduce PCS2 (-8, -7 and -6) and PCS12 (-8) point mutations procedures described by manufacturer (Figure 8B). The presence of all point mutations was confirmed by direct sequencing (Figure 8C). The BamHI-SphI fragment was cloned back into SIVmac239 to generate the SIVmac239-PCS2 mutant clones; the SphI-XhoI fragment was cloned into the SIVmac239 to generate both SIVmac239-PCS12 (-8R) and SIVmac239-PCS12 (-8E) clones (Figure 8D).
A. SIVmac239 pSP73 Ligation

B.

1. Mutant Strand Synthesis
   Perform thermal cycling to:
   - Denature DNA template
   - Anneal mutagenic primers containing desired mutation
   - Extend and incorporate primers with high-fidelity DNA polymerase

2. DpnI Digestion of Template
   Digest parental methylated and hemimethylated DNA with DpnI

3. Transformation
   Transform mutated molecule into competent cells for nick repair
Figure 8. Construction of major PCS amino acid mutations. (A) Digestion of both SIVmac239 and pSP73 vector with BamHI and SphI or SphI and XhoI followed by ligation using T4 DNA ligase to create pSP73-PCS2 and pSP73-PCS12, respectively. (B) Site-directed mutagenesis strategy to create the point mutations. (C) Sequencing alignments to confirm the successful introduction of each individual mutant into the 5 SIVmac239. A) PCS2-8, B) PCS2-7, C) PCS2-6, D) PCS12-8E, E) PCS12-8R. (D) Full length SIVmac239 PCS mutant clones.
Once all the mutations were confirmed correct in the new full length mutant virus, we produced infectious virus stocks by transient transfection of 293T cells with SIV$_{mac239}$ wild type or each PCS mutant plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All 293 T cells were transfected at 85% confluence, virus-containing supernatant were collected 48 hrs. after transfection. The supernatants were clarified centrifugation and then frozen at -80°C for titration and virus RNA level quantitation. These stocks are being titrated by TCID50 analysis for use in competitive fitness evaluations.
**FUTURE WORK**

*Competitive Fitness Assay-*

To continue the evaluation of the PCS vaccine on SIVmac239 fitness, competitive fitness assays will be carried out as described by Anastassopoulou *et al.* (Anastassopoulou *et al.*, 2007). However, we have adapted the described HIV-specific assay for use with SIV isolates. In selecting the appropriate assay for viral fitness determinations, there were a number of limitations and concerns. A few of these experimental decisions are shown below (*Figure 9*). For example, using conventional RT or p24 antigen capture assays to distinguish two similar SIV isolates in a single culture is not currently feasible (Quiñones-Mateu *et al.*, 2000).

*Figure 9.* Practical considerations in the design of a competitive viral fitness assay. Schematic representation of each of 5 aspects to be considered in the selection of the most appropriate assay conditions including Culture environment 1, Virus isolate 2,
Inoculum titration 3, Assay specifics 4, and Experimental endpoints 5. Adapted from Quinones-Mateau and Arts, 2006.

However, it is possible to make this distinction using a comparator virus that contains a unique region that can be used to differentiate itself from other virus isolates. In order to provide a more accurate portrayal of *in vivo* viral fitness a competitive fitness assay using mutant SIVmac239 and wild type SIVmac239, which represents the founder virus, was developed based on the assay used by Anastassopoulou *et al.* (Anastassopoulou *et al.*, 2007). In this assay real time TaqMan quantitative PCR (QPCR) is used to quantify competing viruses using probes that differentially target silent mutations artificially created on the *vif* gene. Our assay will consist of a wild type SIVmac239 containing silent mutations to be our comparator virus versus the SIVmac239 clones containing mutations in inserted in to the PCS2 and PCS12 fragments by site-direct mutagenesis.

![Figure 10. General setup of a virus competition assay.](image)
Fitness (W) can be calculated by the proportion of virus in a dual infection (f_o) divided by its initial level in the inoculum (i_o). The relative fitness difference (W_{diff}) can be determined by the ratio of the more fit (W_m) and less fit (W_L) virus. Figure adapted from Quinones-Mateau and Arts, 2003.
DISCUSSION

This study was initiated to examine the effectiveness of a novel vaccine strategy targeting viral PCS. The three main goals of this study are first to identify prominent amino acid mutations around PCS region that were thought to be associated with selective pressure elicited by an experimental vaccine, secondly to create SIVmac239 clones containing PCS mutations selected by immune pressure and finally to assess the comparative fitness of these mutant clones to a wild type SIVmac239, which would represent a founder virus.

Vaccines Targeting Protease Cleavage Sites

HIV-1 is an incredibly dynamic virus that uses a high mutation rate and rapid replication cycle to effectively escape host immune responses (Barouch, 2013; Coffin, 1995). Coupled with the ability to integrate into a host cell genome and become latent, rendering the virus invisible to the immune system, HIV-1 has demonstrated that conventional vaccine concepts may not be capable of preventing infection and eradicating active infection. Most of the previously described vaccine attempts are examples of conventional concepts that try to stimulate broad, potent antibody responses to known HIV-1 antigens, such as Gag, Pol and Nef peptides. However, the classical vaccine approach to elicit either T cell or antibody responses against HIV-1 has failed so far or produced a modest effect. Therefore, new approaches and ideas are needed to develop novel and effective vaccines.
The PCS targeting vaccine being evaluated varies from previous vaccine attempts by looking into the natural immunity seen in ECs and LTNPs for insights into fighting the virus. Several studies have revealed that a key difference between LTNPs and HIV+ individuals who progress to AIDS involves the degree of immune activation and specificity of immune response (Alimonti et al., 2006; Fowke et al., 1996; Mogensen et al., 2010; Zaunders & van Bockel, 2013). Instead of relying on antibody-mediated humoral immunity, many LTNPs possess specific HLA alleles corresponding to specific peptide epitopes that appear to effectively control HIV pathogenesis via cell-mediated processes. The degree of system wide immune activation is also believed to play a pivotal role in the progression to AIDS (Mogensen et al., 2010). Chronic immune activation is thought to cause “exhaustion” of the immune system, lessening its effectiveness, while simultaneously providing more targets for HIV infection through the systemic proliferation response caused by chronic immune activation.

This PCS vaccine differs by targeting very specific, highly conserved PCS that are known to have immunogenic effects mediated by many prevalent HLA class I alleles. By generating a specific, focused immune response this vaccine could potentially avoid the detrimental side effect of “over immune activation”. In addition, the vaccine could also force the mutation of conserved sites that are essential for viral replication, rendering the virus less fit and pathogenic. Viral fitness has been shown to be a critical factor in determining viral set point and rebound, therefore, any therapy capable of forcing the mutation of less fit virus would be worth exploring (Trkola et al., 2003).
Continuing the on-going characterization of viral fitness in the context of vaccine-elicited immune pressure is critical for designing an effective vaccine concept to combat HIV-1. Competitive fitness assays allow researchers to more accurately measure the effectiveness of vaccines by creating an environment that is more similar to the *in vivo* environment in an active infection. Although there will always be limitations to the value of *ex vivo* assays compared to *in vivo* mimicking the intra-species competition that exist from *in vivo* viral swarms is a step in the right direction.

In addition to the mutants we created, harboring single mutations, we plan to create mutant viruses harboring the various combinations of mutations possible. This will assess whether multiple PCS mutations have a multiplicative effect on fitness and infectivity of virus. We also plan to further describe the molecular characteristics of these mutant viruses using ELISA and Western Blotting techniques.

With all of the encouraging results and the knowledge gained from this pilot study, we will be able to provide pivotal information to develop an effective HIV vaccine that selectively target the key sites of HIV-1 such as PCS and prevent from HIV-1 acquisition.
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CURRICULUM VITAE

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Birth Year: 1990

EDUCATION

Boston University, Graduate School of Medicine, Boston, MA
Master of Science, Medical Sciences, Anticipated May 2015

The College of William & Mary, Williamsburg, VA
Bachelor of Science, Biology, May 2012

RESEARCH EXPERIENCE

Beth Israel Deaconess Medical Center, Center for Virology and Vaccine Research, Boston, MA
Graduate Research Assistant, 2008-2009
Principal Investigator: James B. Whitney, Ph.D
Completed Master’s Thesis research which consisted of comparing the relative fitness of SIV harboring the prominent mutations of virus isolated from Cynomolgus macaques exposed to a novel vaccine and wild type SIVmac239. Also contributed to other projects in the lab which focused on the characterization of SIV viral dynamics, HIV/SIV reservoir maintenance and the development of novel HIV eradication techniques.

College of William and Mary, Department of Biology, Williamsburg, VA
HHMI Freshman Research Scholar, 2008-2009
Advisor: Professor Mark Forsyth
Selected to participate in a research program investigating the genome architecture and evolution of mycobacteriophages. Contributions consisted in finding, isolating, mapping and analyzing an unknown mycobacteriophage’s genome. This work resulted in the publication “Expanding the Diversity of Mycobacteriophages: Insights into Genome Architecture and Evolution” published in PLOS ONE.

LEADERSHIP/ SERVICE

Latino Medical Student Association (Boston University Chapter), Boston, MA
Executive Board Member, January 2013- Present
The goal of the Latino Medical Student Association is to raise awareness about health issues faced by the Latino population, as well as, creating service opportunities for students interested in working with the Latino community. Responsible for selecting,
organizing and executing event opportunities on/off the medical campus to fulfill the organization’s goals.

Kardiozentrum and Hospital del Niño de La Paz (La Paz Children’s Hospital), La Paz, Bolivia
Intern, March 2013- May 2013
Learned different techniques used to determine congenital heart defects on children. Counseled patients on various therapies. Observed surgeries, including one in which a stent was placed in the heart of a child with patent ductus arteriosus.

Hospital Obrero, La Paz, Bolivia
Intern, March 2013- May 2013
Learned about the various facets of a general surgeon role by shadowing Dr. Claros, a general surgeon at a state run hospital. Observed patient diagnoses, pre/post operation consultations, and morning rounds. Attended an average of 2 surgeries per week.

Beach Health Clinic, Virginia Beach, VA
Volunteer, December 2012- March 2013
Transcribed medical histories, notes on current examinations and future treatments while shadowing several types of physicians (Podiatrists, Internists, and Neurologists) in the examination rooms. Conducted routine clinical tests, and assisted the administrative staff by maintaining/creating patient medical files, and screening new patients. The Beach Health Clinic is a non-profit free clinic that treats the underserved and homeless community in Virginia Beach, VA.

Virginia Beach General Hospital, Virginia Beach, VA
Volunteer, December 2012- March 2013
Assisted people coming into the ER with minor triage, including giving directions and comforting the patients and families in the waiting room.

Balderrama Clinic, Pasadena, CA
Volunteer, Summer 2011
Transcribed medical histories and triage reports under the supervision of Drs. Erik and Frank Balderrama
The Balderrama Clinic is a clinic in Pasadena, CA that serves the predominantly Latino local community.

PROFESSIONAL EXPERIENCE

OSLE Properties, Virginia Beach, VA
Property Manager, July 2010 – Present
Oversee tenant selection, lease agreements, property repairs/maintenance, rent collection and tours of the property for prospective tenants.
Hampton Inn Historic, Williamsburg, VA
Customer Relations Representative, July 2008 – June 2010
Responsible for all services provided by the hotel to the guest. This included creating/modifying reservations, greeting and accommodating to the needs of hotel guests at a 100% satisfaction guaranteed service level.

LANGUAGE SKILLS
Read, write, and speak Spanish fluently
Read, write, and speak French - basic

AFFILIATIONS
Latino Medical Member Association (BU), Executive Board, 2013 - present
Lambda Chi Alpha, Member 2010-present

Publications:
• “Expanding the Diversity of Mycobacteriophages: Insights into Genome Architecture and Evolution” - published in PLOS ONE

Athletics
• U17 World Cup Qualifiers, Trinidad & Tobago – Puerto Rico (PR) National Soccer Team, 2006