DEVELOPING A BROADLY REACTIVE HIV ENVELOPE VACCINE

by

Hermancia Sulvina Eugene

B.S. Biology, Cameron University, 2005

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Hermancia Sulvina Eugene

It was defended on

April 11, 2012

and approved by

Karen A. Norris, Ph.D, Professor, Department of Immunology

Clayton Wiley, M.D., Ph.D, Professor, Department of Pathology

Robert. L Hendricks, Ph.D, Professor, Department of Immunology

Phalguni Gupta, Ph.D, Professor, Department of infectious Diseases and Microbioology

Jodi. K. Craigo Ph.D, Research Associate Professor, Department of Microbiology and Molecular Genetics

Dissertation Advisor: Ted Ross, Ph. D, Associate Professor, Department of Microbiology and Molecular Genetics

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Hermancia S Eugene, Ph.D

University of Pittsburgh, 2012

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One of the challenges facing HIV vaccine development is the diversity of the envelope protein (Env). Env only vaccines protect against homologous virus infection, but have not protected from heterologous virus infection. In this project, soluble Env trimers were designed and constructed to elicit anti-Env immune responses that recognize contemporary viral isolates. Env sequences isolated from the Americas, Europe, Africa, and Asia were aligned to generate a consensus Env sequence that represents clades A, B, C, E, and group M. These Envs were truncated at the transmembrane domain (Env_{gp140}) and stabilized with a T4 fibronectin domain from bacteriophage. The overall hypothesis is a multi-clade vaccine expressing HIV-1 envelope proteins in their trimeric structure will elicit a broad cross-reactive immune response capable of protecting from heterologous virus challenge. Two vaccine strategies were used: a polyvalent vaccine strategy, using a mixture of equal amounts of consensus Env trimers of clades A, B, C and E (Poly consensus) in equal amounts, was directly compared to a single consensus Env representing the entire group M (Con M). Initial immunogenicity studies conducted in mice showed consensus envelopes to be immunogenic. These studies were followed by immunogenicity and efficacy studies in the rhesus macaque model. The macaques were

immunized intramuscularly three times with equal amounts of total protein of either the polyvalent or consensus vaccine in combination with Imject® alum. Both vaccines were immunogenic in monkeys and sera from vaccinated animals detected and neutralized the same number of envelopes from HIV-1 isolates. After challenge with the simian-human immunodeficiency SF162p4 virus, all polyvalent vaccinated animals were infected and two of the four animals vaccinated with Con M Env_{gp140} showed no evidence of infection. After CD8+ T depletion of the two aviremic animals, virus emerged in one of those animals indicating potential CD8+ T cell viral control. The other animal vaccinated with Con M Env_{gp140} remained aviremic. The presence of cross-binding antibodies and low neutralizing antibodies elicited by two multi-clade vaccines did not provide protection to all vaccinated animals.

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PREFACE

I would like to thank my immediate family ***Maggie Eugene, Pius Eugene, Magdalene N. Eugene*** and ***Ascencia N. Eugene***. I would also like to say a special thanks to ***Wayne Zamore*** for choosing to make this journey with me. Thanks to my support network both in the US, (Pittsburgh, Oklahoma, New York, Indiana, North Carolina, Sacramento and Colorado), St.Lucia and Dominica (***The Zamore family***). I could always count on the support and love of every one of you through the years. I thank God for your presence in my life. Though you were not always here physically, your love and concern for my success was always felt. Love you all!!

1.0 INTRODUCTORY CHAPTER

Immunotherapies and Vaccines

This chapter was modified with Permission from:

Hermancia S. Eugene and Ted M. Ross (2011).

Immunotherapies and Vaccines, HIV and AIDS

Updates on Biology, Immunology, Epidemiology and Treatment Strategies,

Nancy Dumais (Ed.), ISBN: 978-953-307-665-2, InTech,

Available from: http://www.intechopen.com/articles/show/title/immunotherapies-and-vaccines

1.1 INTRODUCTION

In 1983, Barre-Sinoussi and Gallo in parallel at two independent institutions identified the Human Immunodeficiency Virus (HIV) [3, 4]. The following year, HIV was established as the causative agent of Acquired Immunodeficiency Syndrome (AIDS). After such a monumental discovery, there were expectations that an effective vaccine would soon be available. Unfortunately this expectation has yet to become reality and HIV remains a global epidemic. In 2010, the World Health Organization (WHO) recorded 2.7 million new HIV infections and 34 million individuals living with the virus worldwide [5].

In the developing world most HIV infections occur in individuals between the ages of 25-44 [6]. Consequently, the AIDS epidemic affects the socio-economic development of third world countries [7-10]. In 2000, the world's governments identified AIDS as one of the major hurdles in attaining economic development[10]. AIDS has been associated with social issues such as increased poverty, overwhelmed health systems, decreased life expectancy, increased child mortality and decreased maternal health. In addition, AIDS has made it difficult to eradicate diseases such as malaria and tuberculosis [11-13]. The risk of a healthy individual acquiring TB in their lifetime is 10%, this risk increases to 50% in HIV infected patients.

Due to HIV's impact on global public health and the tremendous economic impact this virus continues to be a global threat. With its discovery ~30 years ago, many areas of research of HIV have evolved. These research areas include basic virology of HIV to host/pathogen response. One way to control infectious diseases is to design prophylactic vaccines. The work recorded in this dissertation aims to add to the knowledge of HIV vaccine development to move the field closer to the goal of a prophylactic HIV vaccine.

1.2 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.2.1 General Background

HIV is a member of the family *Retroviridae* and the genus *Lentivirus*. Classification into the family *retroviridae* is attributable to the reverse transcriptase activity of its polymerase. Its long incubation period during infection, its morphology and its electron dense cylindrical inner core places the virus in the genus *lentiviridae* [14]. The structure and components of a mature HIV virion is illustrated in figure 1. HIV is an enveloped virus about 100 to 120 nm in diameter with an RNA genome approximately 10kb in length. HIV genome contains 3' and 5' long terminal repeats (LTR) and three major genes: group specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) that encode for structural and enzymatic proteins. In addition to the major genes, the genes, *tat, rev, vif, vpr, vpu, vpx,* and *nef* (these will be discussed in detail below) are involve in productive infection (Figure 2).

After the initial discovery of HIV in 1983, a second virus with similar morphology, but antigenically distinct and a 55% difference in sequence homology was identified. This virus was also associated with an AIDS-like illness, and was named HIV-2 [15] . The serological difference observed between HIV-1 and 2 was directed toward the envelope glycoprotein. The antibodies to Gag and Pol of HIV-2 were cross- reactive to HIV-1, but this cross reactivity was not seen with Env antibodies [16]. The first phylogenetic analysis of HIV was based on viruses isolated from Europe, North America and Africa. Initial molecular epidemiology of isolates of HIV-1 was based on sequencing of *env* and *gag*, more recently the analysis of the *pol* gene sequences are used for classification [17] . This analysis led to the establishment of distinct subtypes. For a new viral isolate to be classified as new clade it must be sequenced from three

individuals with no history of relation or epidemiological relation. The diversity seen in HIV particularly HIV-1 is tremendous. HIV-1 is classified into three major groups: Main (group M), Outliers (group O) and non-M , non O (group N) (Figure 3). HIV-1 diversity has expanded with recently newly identified group P [18]. Members of the main group of HIV-1 (Group M), which are associated with the vast majority of infections, are further divided into clades or subtypes, A-D, F-H and J-K. HIV-1 group O isolates have been recovered from individuals living in African countries Cameroon, Gabon and Equitorial Guinea[19, 20]. In addition, isolates from different clades can recombine forming circulating recombinant forms (CRFs). CRFs develop as a consequence of a single cell being infected by genetically distinct viral strains and template switching between two different viral RNA during the reverse transcription reaction. Group M viruses are responsible for more than 95% of HIV infection worldwide. The diversity seen in the group of viruses is seen in the *gag* gene (15-20%) and *env* (20-30%) between clades and *gag* (3%-10%) and *env* (5-15%) within clades. Within an infected person the envelope diversity has been reported to be up to 10%. This level of genetic diversity is one of the major challenges facing the development of a preventative vaccine.

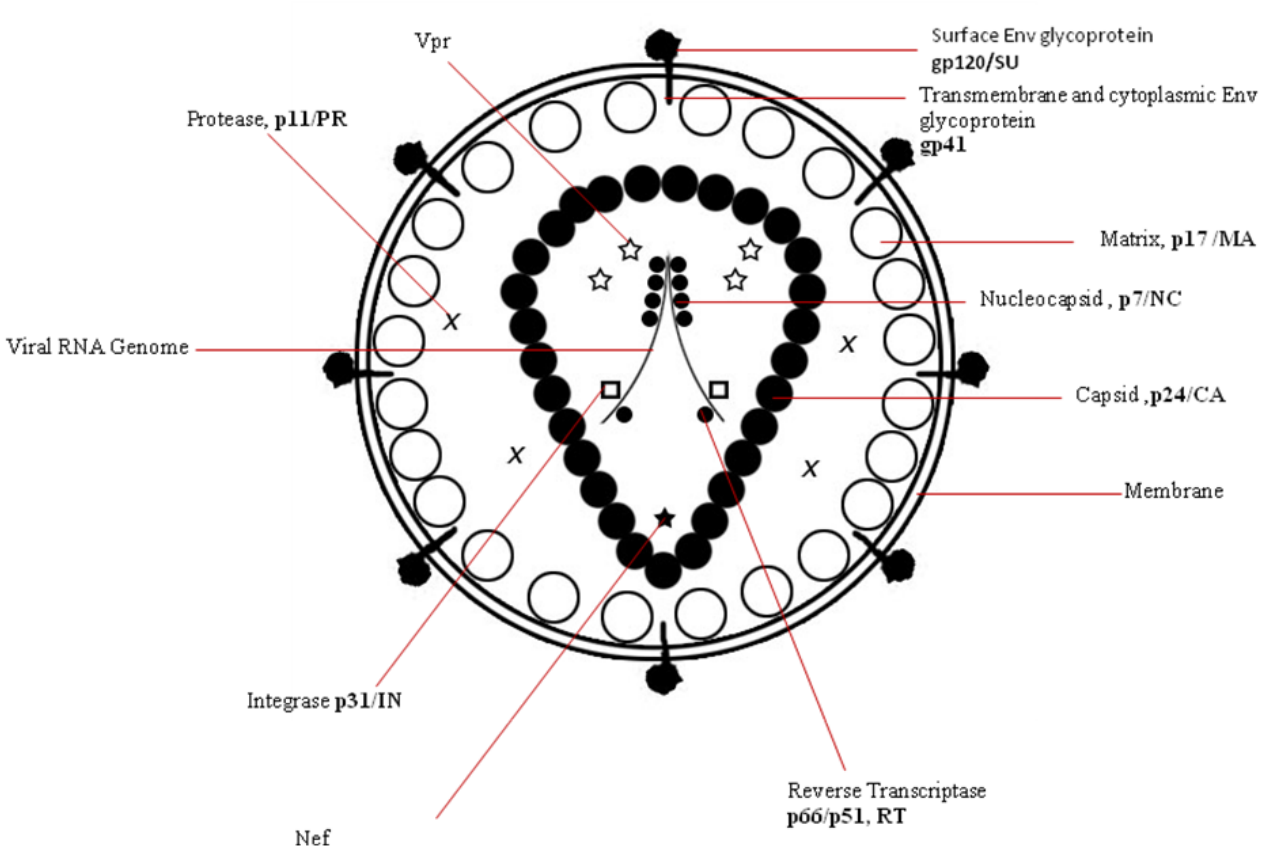

Figure 1: Mature HIV virion.

(image reference: David M.Knipe et. al. Fields Virology vol.2)

The viral glycoprotein Env is present as trimeric spikes on the surface of the viral lipid envelope. The structural protein Gag after protoelytic cleavage give rise to the viral matrix, nucleocapsid (bound to the RNA genome) and the viral capsid. The viral genome, enzymatic proteins (integrase and reverse transcriptase), and accessory proteins are found within the capsid.

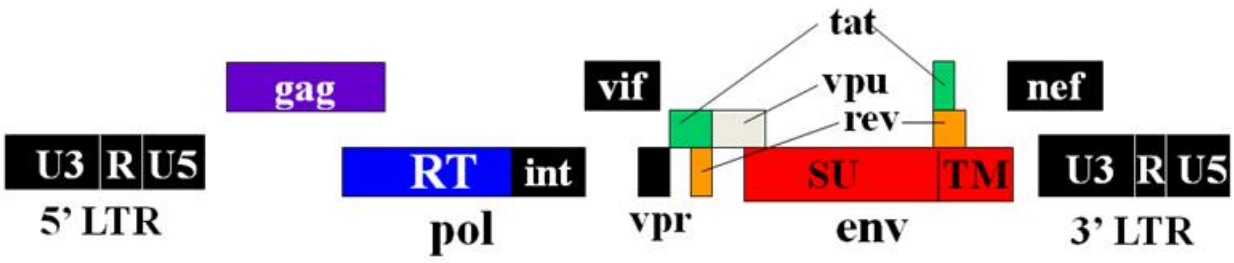

Figure 2: HIV Genome

The long terminal repeats (LTR) are sites for initiation of viral RNA synthesis and required for proviral integration into the host cell genome. The structural and enzymatic proteins are coded by gene *gag, pol* and *env*. The regulatory proteins are coded by genes *tat* and *rev,* and the accessory genes coated by gene products *vpr, vif, vpr, nef*.

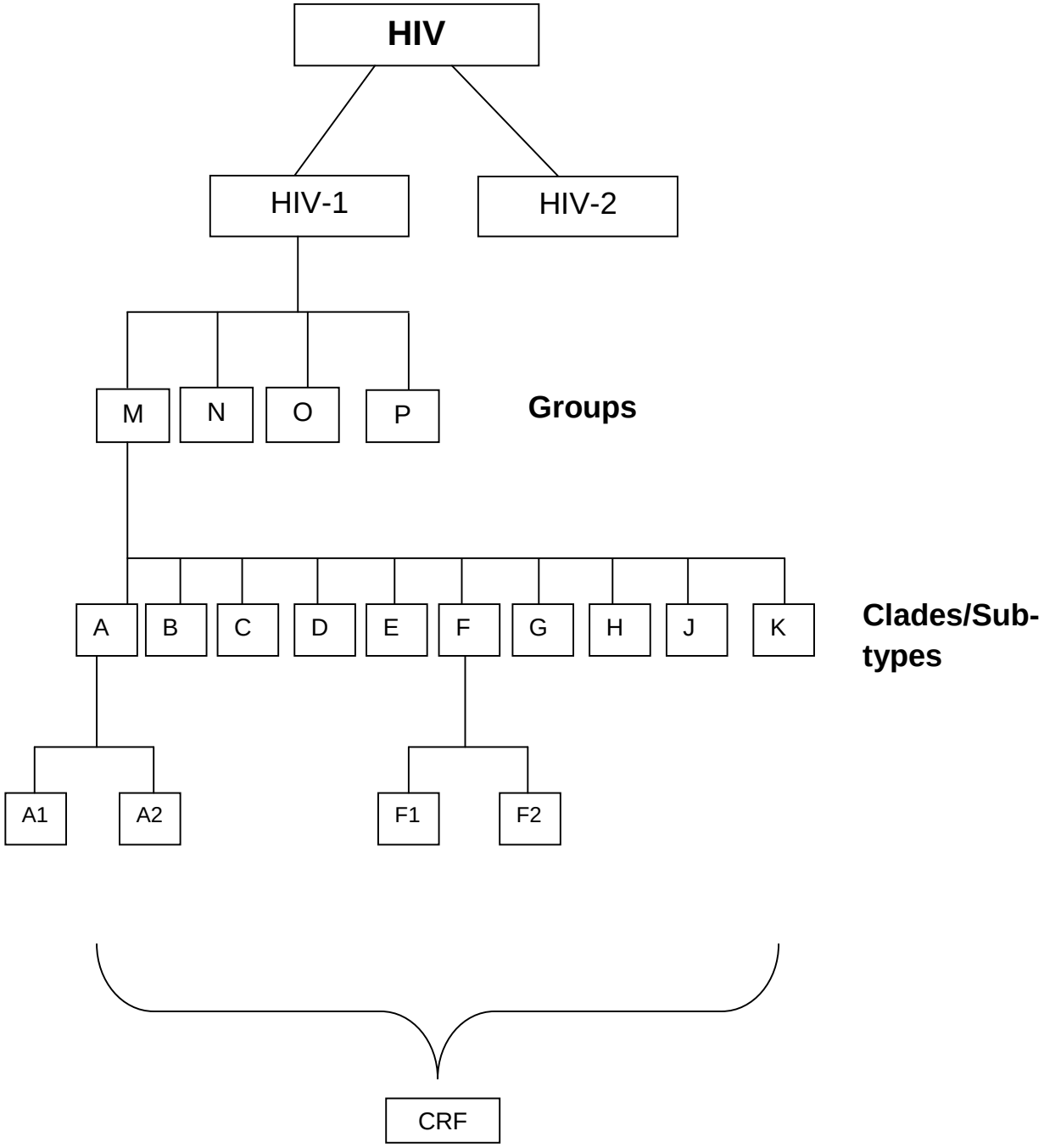

Figure 3: HIV Taxonomy

HIV is classified into HIV-1 and HIV-2. HIV-1 is then divided into groups M, N and O. Group M is then divided into clades A-H, J-K. When multiple viruses infect the same cell recombination can occur generating recombinant viruses (CRF).

1.2.2 HIV Proteins

The three major proteins of HIV are Group specific antigen (Gag), Polymerase (Pol) and Envelope (Env). Gag encoded by *gag* gene forms the structural elements of the virus. Gag contains p24 that encodes for the viral capsid protein, p17 that encodes the matrix protein, p6 and p7 that encodes nucleocapsid proteins. The numeric values given to these proteins represents the size at which they migrate on a denaturing protein gel. Polymerase encodes the proteins reverse transcriptase, intergrase and protease. Reverse transcriptase is common to all retroviruses and transcribes vRNA into dsDNA. Integrase integrates the viral dsDNA into the host DNA. Protease is needed for the cleavage of gag and pol proteins into individual proteins. Protease plays a vital role in virus maturation as new viral progeny bud from infected cells. Envelope encodes for the precursor gp160 monomer which are made up of gp120 and gp41. The gp120 is the external domain of the glycoprotein which interacts with the CD4 receptor and co-receptors (CCR5 and CXC4). The gp41 unit of the glycoprotein functions to fuse the cell and viral membrane. The envelope's functional unit is a trimer.

HIV also contains accessory proteins involved in replication, infectivity and combating host immune system. Tat enhances processivity of the viral polymerase and facilitates elongation of vRNA. Rev regulates mRNA production. It facilitates the transport of mRNA unspliced or singly spliced into the cytoplasm by binding to Rev-responsive element. During infection Nef reduces MHC I, CD4, CD8 expression on cell surface, affects T-cell activation, enhances virion infectivity, increase and decrease virus replication. Vpu affects viral release, disrupts Env-CD4 complex, and facilitates CD4 degradation. Vif increases virus infectivity, affects virion assembly/or viral DNA synthesis, and is the antagonists of cellular proteins CEM15/APOBEC3G. Cellular proteins such as APOBEC3G evolved in primates to interfere

with SIV and HIV viral replication. Vpr causes G_2 arrest and facilitates entry of the pre-integration complex into the nucleus. Vpx performs the same functions as Vpr but it is only found in HIV-2.

1.2.3 HIV Envelope

The viral envelope is responsible for viral binding and entry [21, 22]. Due to its vital role, the envelope is a good target for vaccine design. The monomer of the viral glycoprotein consist of a heavily glycosylated exterior portion referred to gp120 and the transmembrane glycoprotein referred to as gp41 (Figure 4). The Env forms a trimer complex on the surface of the virus. The gp120 is responsible for binding the receptor and co receptor CD4 and CCR5 respectively. Based on sequence alignment, the gp120 unit of envelope consists of five genetically conserved and five genetically variable regions [23]. The gp41 transmembrane domain has of an ectodomain and a cytoplasmic domain. The ectodomain consists of the N-terminal fusion peptide and the two heptad repeats (HR1 and HR2) which are involved in cellular and membrane fusion[23]. This ectodomain is found beside a membrane spanning domain and cytoplasmic domain predicted to have to helix motifs. The gp120 and gp41 units of envelope are linked by a noncovalent bond. The cleavage site between the gp120 and gp41 subunits contains a furin cleavage site amino acid sequence Arg-Glu-Lys-Arg [24]. Besides being involved in viral entry, HIV Env contain epitopes that elicit immune responses that important in both vaccine development perspective and diagnostics.

Figure 4 Schematic of HIV envelope

The Env_{gp160} is composed of two subunits gp120 and gp41. The subunit gp120 includes five constant and five variable regions. The gp120 is responsible for binding to HIV cellular receptors. The V3 domain has been reported as the major determinant in co-receptor binding, with regions V1, V2, C4 and gp41 able to modulate its tropism. The subunit gp41 is responsible for membrane fusion. Together these two subunits in envelope spike on the virus facilitate viral entry into susceptible cells.

1.3 HIV VIRUS REPLICATION AND CLINICAL PROGRESSION AND TREATMENT

1.3.1 HIV Replication

The initial step in HIV replication is the binding of the virus envelope to its primary receptor CD4 (Figure 5). This initial binding results in a conformation change in envelope resulting in the availability of the co-receptor binding site. The human CCR5 or CXC4 are the co-receptors used by HIV-1. Co- receptor binding is followed by viral and cell membrane fusion facilitated by the gp41 subunit of Env protein.

Fusion of lipid bilayers allows the release of the viral capsid into the cell. The viral RNA genome is reverse transcribed by the viral reverse transcriptase in the partially uncoated capsid. The partially double stranded DNA complex (provirus) bound by viral nucleoproteins is called

pre-integration complex (PIC). The PIC enters the nucleus by the viral protein vpr. The provirus inserts into the host cell genome by the virally encoded integrase.

In the infected cell, the provirus is used as a template by cellular Polymerase II (Pol II) to directly synthesize three major viral messenger RNA (mRNA) species. The processivity and efficient synthesis of viral messenger RNAs is facilitated by the viral Tat protein that interacts with the secondary TAR structure found in the nascent RNA. The viral mRNA is then transported by the viral protein Rev from nucleous into the cell's cytoplasm for protein translation. The Env protein is translated from RNA by ribosomes bound to endoplasmic reticulum and goes through the secretory pathway for post translation modification and oligomerization. The Env trimer is transported to the plasma membrane for incorporation into virion.

The Gag and Gag-pol proteins and other cellular proteins are translated on free ribosomes in the cytoplasm. The N-myristic acid moiety of the Gag protein allows trafficking of Gag and Gag-pol protein to the plasma membrane. At the plasma membrane the Gag and Gag-pol associate with the newly synthesized RNA genome to form a condensed capsid that buds from plasma membrane. As the immature virion buds from the cell, the viral protease is incorporated into the virion and cleaves the Gag-Pol protein to form a mature virion.

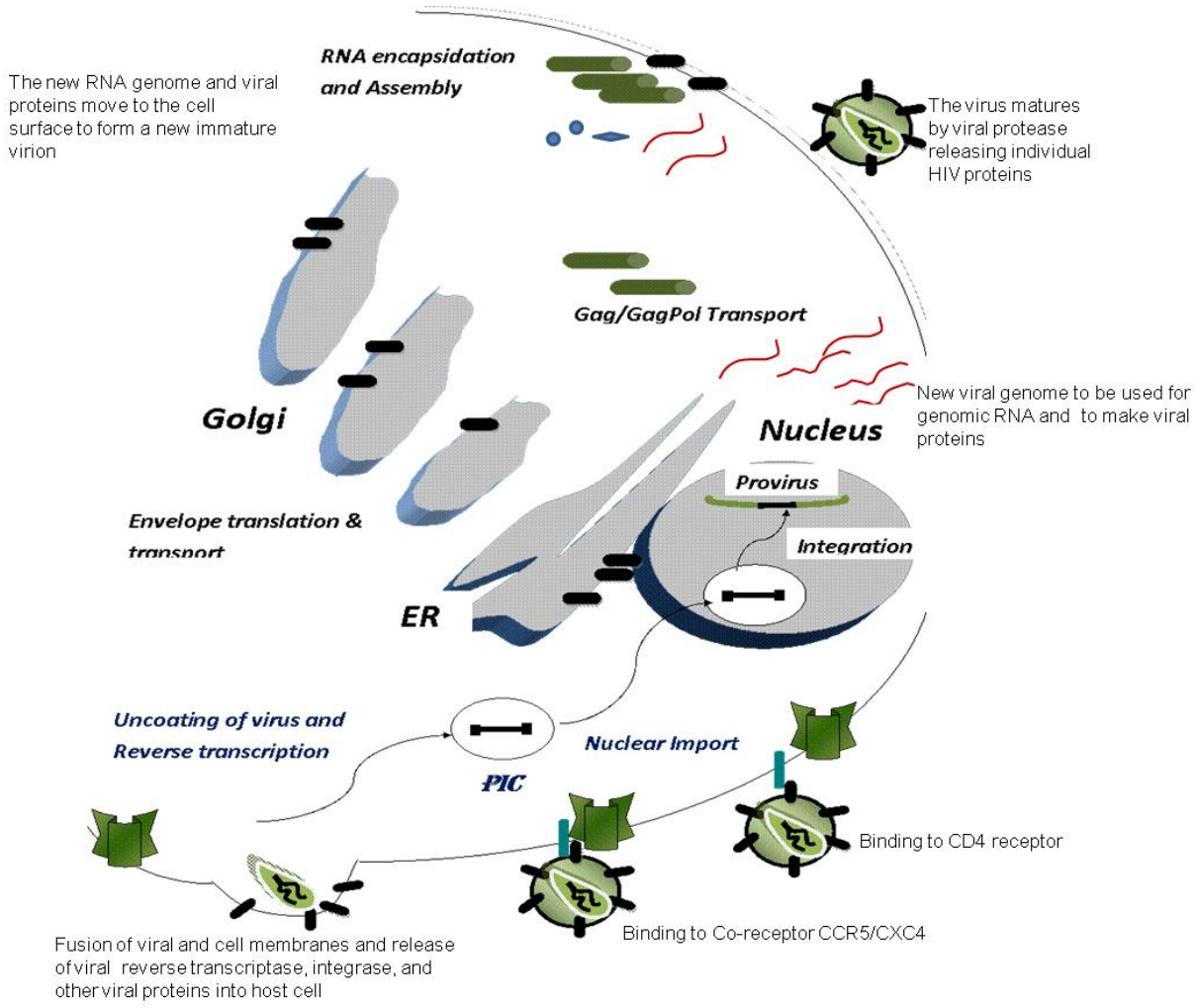

Figure 5: HIV replication cycle

Infection begins with Env mediated entry through binding with receptors CD4 and CCR5. This is followed by membrane fusion and capsid release. Partial capsid uncoating allows for the initiation the RNA genome transcriptions into DNA. The viral DNA is then integrated into the host genome. Viral RNA is then synthesized using the integrated DNA (provirus) and cellular polymerase II. The RNA products are used for protein translation and incorporation into the virus. The Env is translated and processe through the secretory pathway and directed to the cell membrane. The Env, Gag/Pol and other viral proteins are transported and assembled into immature virion cell membrane. After budding the virus matures due to proteolytic cleavage by the viral protease.

1.3.2 Brief Review of HIV/AIDS Clinical Progression

A characteristic of HIV infection is decreased number of $CD4^+$ T helper cells. This decrease in CD4+ T cells is partly due to viral infection and cell apoptosis. In figure 6, the viral loads and $CD4^+$ T cell counts during the HIV/AIDS disease progression is shown. The World Health Organization (WHO) divided the HIV/AIDS progression into four clinical stages [25-27]. During the first clinical stage the patient is asymptomatic and has generalized lymphadenopathy. This phase usually starts with a rapid progression in viral loads for about 3-6 weeks accompanied by a drop in normal CD4+ T cell levels. Normal CD4+ T cell levels are usually between 600-1200 cells per mm^3. About 7-8 weeks after primary infection, the viremia is brought under control by the immune system and a viral set point is established. The established viral set points are dependent on each individual [28]. With the control of viremia, CD4+ T cell levels may recover in some individuals.

During the second clinical stage, CD4+ T cell levels begin to decline accompanied by an increase in viral loads. This clinical stage is characterized by less than 10% weight loss, minor mucocutaneous manifestations such as Herpes Zoster and recurrent upper respiratory bacterial infections. Further decline of CD4+ T cells and increase of viremia is seen as patients enter clinical stage three. Symptoms of clinical stage three include: weight loss of more than 10%, unexplained chronic diarrhea and prolonged oral thrush, pulmonary TB and severe bacterial infections. The last clinical stage, AIDS, usually occurs ten years after primary infection. This phase of symptomatic disease is accompanied by CD4+ T cell counts less than 200 cells per mm^3 of blood and substantial increases in viral loads. Patients are diagnosed with HIV wasting syndrome, and other opportunistic infection such as *Pneumocystis carinii* pneumonia (PCP),

Toxoplasmosis of the brain, herpes simplex virus infection, Kaposi sarcoma, and HIV encephalopathy.

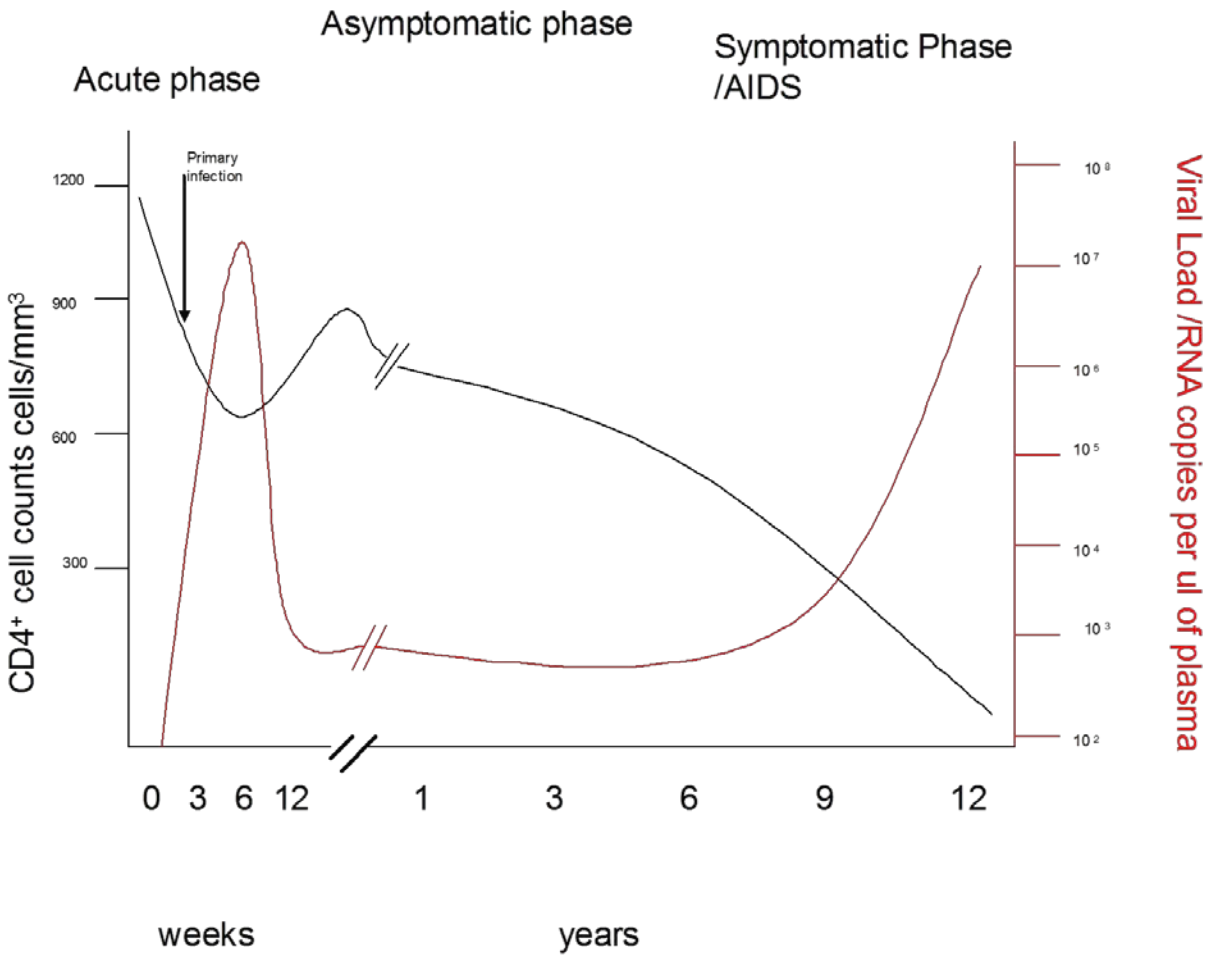

Figure 6. CD4 and viral changes overtime in HIV/AIDS disease progression

During the first six months of HIV infection there is an initial decline of $CD4^{+}$T cells and increase in viral load. This phase of infection is referred to as the acute phase of infection. The acute phase of infection is followed by the asymptomatic phase which is characterize by the restoration of CD4 + T cells and the establishment of viral set point due to control of the infection by the immune system. This asymptomatic phase can last for an average of 10 years until the immune system loses control of the viral infection. This loss of immune control is characterized by CD4+T cell decline and increase in viremia eventually leads to CD4+ T cell counts less than 200 cells per mm3 and diagnosis of HIV wasting syndrome and opportunistic infections.

1.3.3 Highly Active Antiretroviral Therapy

Two years after the discovery of the causative agent of AIDS, the first sign of possible treatment was reported in 1985 with the development of the first antiretroviral compound [29]. This compound was called Retrovir (zidovudine, AZT) and became the first drug in the family of nucleoside reverse transcriptase inhibitors (NRTI). AZT targets the reverse transcription process of HIV replication cycle. AZT is a nucleoside analog, during reverse transcription the analog competes with the natural Deoxyribonucleic acid resulting in strand termination. Since AZT, several NRTI and other families of drugs targeting the replication cycle of HIV have been discovered. As of 2010, the FDA has licensed twenty-five antiretroviral drugs [30, 31]. These drugs can be grouped based on the mode of action and are placed into one of the following groups: nucleoside reverse transcriptase inhibitors (NRTI), nucleotide reverse transcriptase inhibitor (NtRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), co-receptor inhibitor (CRI), integrase inhibitor (INI), and fusion inhibitor (FI) [32]. Figure 7 highlights licensed drugs and the mode of action of each group of drugs.

In 1996, the first use of combination drug therapy was attempted using a protease inhibitor that was combined with an NRTI [33]. Combinational therapy confirmed that there was a longer period of undetectable or reduced viral loads as well as recovery of CD4+T cells in the blood. Combination therapy or HAART is now the treatment of choice for HIV infected individuals and results in various clinical outcomes [34, 35]. Individuals on HAART need to be monitored at all times to ensure the combinational therapy is effective and does not result in viral rebound and generation of escape mutants. Individuals on HAART will need their treatment regimen adjusted upon development of viral resistance [36, 37]. In addition to viral resistance, HAART is highly toxic to patients resulting in reduced patient compliance [38, 39]. Moreover,

HAART treatment is expensive and therefore people living in developing countries have less access to drugs, even though these are the epidemic locations. Based on surveys and clinical research, the WHO has identified factors that need to be considered to determine HAART treatment in an adult [40]: 1) suitability of drug combination, 2) licensing of drugs by national regulatory department and recommended dose, 3) toxicity profile of the drug, 4) availability of laboratory monitoring, 5) potential of maintenance and adherence to treatment, 6) prevalence of co-existing infections (*e.g.* tuberculosis), 7) child bearing age, 8) availability of local and international manufacturers, and 9) price and effectiveness of drug.

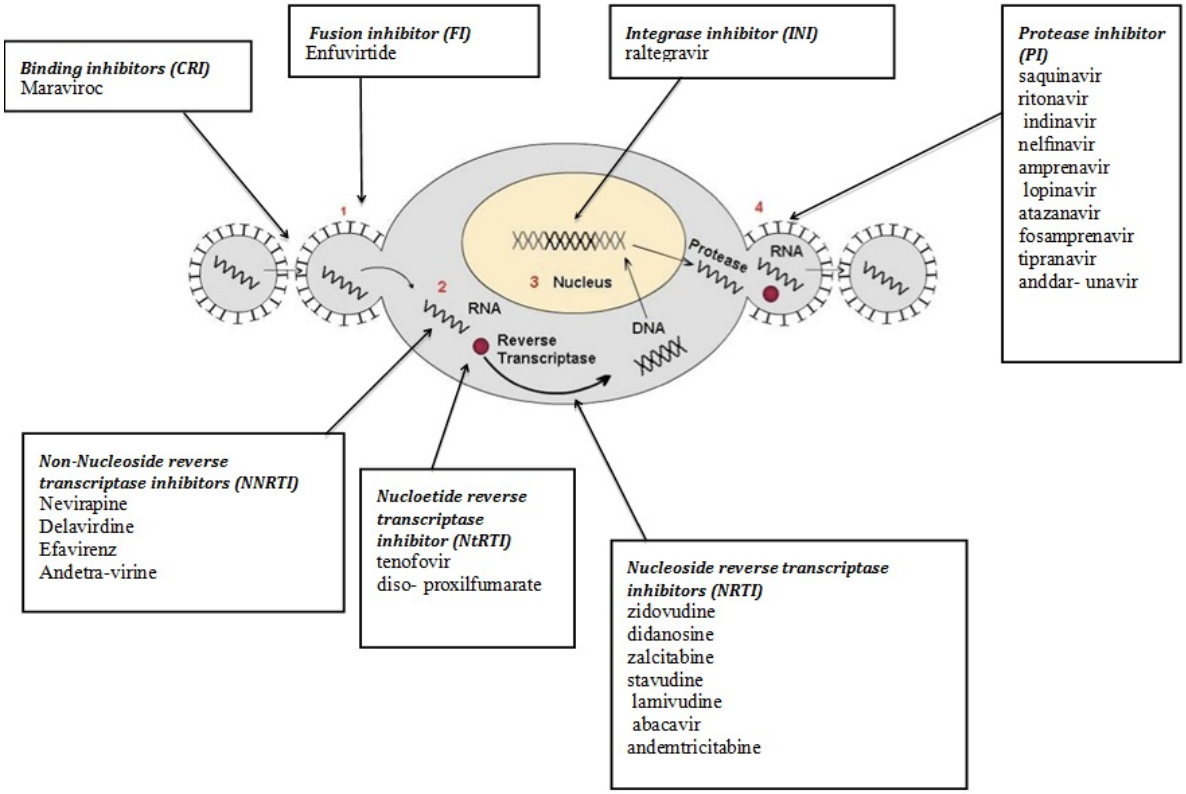

Figure 7: Diagram showing licensed antiretroviral HIV drugs.

(JM Kilby - Expert opinion on investigational drugs, 1999 - informahealthcare.com)

This schematic highlights licensed drugs used in HIV treatment and the step of replication they target.

One of the complications that an individual can experience on HAART is immune reconstitution inflammatory syndrome (IRIS) [41]. The hallmark of IRIS is a contradictory worsening of an existing infection or disease process or the appearance of one soon after initiation of therapy [42]. It is the result of unbalance reconstitution of effector and regulatory T cells, leading to an uninhibited inflammatory response in patients treated with ART. IRIS is usually associated with fungi, herpes viruses and mycobacterial infections. IRIS occurs in individuals recovering from immunodeficiency. Criteria for IRIS are: 1) Response to antiviral therapy by: viral loads >1 $\log_{10}$/ml decrease in RNA level, 2) symptoms cannot be alleviated by: clinical course of treatment, medication side effects or toxicity, treatment failure or complete non adherence [43]. IRIS is also recorded in individuals with HIV co-infections such as tuberculosis [44]. Additionally, individuals on HAART develop other diseases such as cardiac and metabolic complications that are affiliated with aging [45]. The side effects of HAART treatment and limited availability in HIV endemic areas are the drive for development of new immunotherapies for HIV.

1.4 HIV VACCINE DEVELOPMENT

1.4.1 Viral and Immune Challenges

Several factors have contributed to the delay of HIV vaccines and therapeutics. These factors can be grouped into two main categories: 1) intrinsic viral characteristics and 2) viral and host interactions. The intrinsic viral properties of HIV, such as rapid replication, virus mutation, virus recombination and viral integration, have been obstacles in drug and vaccine development [46,

47]. One of the major problems in HIV vaccine development is the high sequence variability of viral isolates [48, 49]. The classification of HIV into clades is covered in the HIV nomenclature proposal now found on the Los Alamos HIV Sequence database website [50]. A major contributor to the high variability of the virus is the lack of proof reading activity present in the viral polymerase (reverse transcriptase) combined with rapid replication rate. Such a combination allows for emergence of viral isolates that can evade the immune response elicited to older viral sequences. The constant escape from immune surveillance results in a constant need for "catch up" by the immune response. Another reason for increased variability is the ability of the virus to genetically recombine [51]. Due to the possibility of superinfection, (a new infection occurring in a patient having a preexisting infection) viruses from different clades can be present in the same cell during replication and may result in recombinant viruses. For example, a virus classified A/E has an envelope derived from a clade A virus and Gag proteins derived from a clade E virus. The other major viral property that works against effective therapy and viral clearance is viral integration. HIV contains a viral integrase responsible for integration of the HIV provirus into the DNA of an infected cell [52]. Provirus integration is an essential part of replication [53]. This integrated viral DNA results in both establishment of viral reservoirs in the host and disruption of the immune responses against the infecting virus [54-57]. These viral reservoirs are a source of actively replicating viruses in individuals who have controlled infection and have undetectable levels of virus [58-60]. HIV reservoirs contribute to the lack of virus eradication and the need for continuous HAART therapy by HIV infected individuals to prevent virus rebound [61]. Latent reservoirs allow the virus to persist as genetic information in the form of a stable integrated provirus. Therefore, poor compliance and inadequate suppression therapy may lead to drug resistant viruses and limiting treatment options.

Viruses in these reservoirs are genetically similar and devoid of resistant viruses. Even though the latent reservoirs guarantee lifelong viral persistence, HAART therapy can suppress viral evolution over time as long as the toxicity induced by the regimen can be overcome.

The lack of a defined correlate(s) of protection for HIV is a major obstacle in vaccine and therapeutic development. Humoral immune responses were initially proposed as a correlate of protection. Passively administering anti-HIV antibodies to experimental animals results in protection from infection [62-64]. The first prophylaxis vaccine to enter phase III trials, AIDSVAX by VaxGen, induced antibodies to HIV vaccine envelopes, but the vaccine was not efficacious [65]. The failure of the initial studies brought into question the role of humoral responses as the correlate of protection. The antigens used in these studies, monomeric gp120 and monomeric gp160, are not the functional unit of the HIV envelope. The HIV envelope is trimeric on the surface of the virus particle. Studies using trimeric envelope immunogens improve the induced humoral responses [66-68]. Also, the recent vaccine trial in Thailand (RV144) provided data to support the possibility that the presence of binding antibodies to envelope may be the HIV correlate of protection (the RV144 clinical trial will be discussed later) [69].

Many investigators have designed preventative vaccines for HIV that induce immune cellular responses [70-72]. Data from preclinical studies, as well as infected individuals, show that an effective cellular response was able to control viral replication and resulted in reducing progression to AIDS [73, 74]. Coming on the heels of failed humorally-driven trials, the certainty of developing a preventative vaccine is questioned and preventing progression to disease by cellular immunity is proposed as an alternate focus. Vaccines aimed at eliciting cellular responses for preventing infection or disease progression have also not been successful.

In 2007, the Merck HIV vaccine trial used adenovirus to deliver HIV genes *gag, pol* and *nef*. The trial was stopped after intermediate data analysis showed no supportive evidence to continue [75]. It appears that pre-existing immune responses to the adenovirus may have increased susceptibility to infection. The immune correlate(s) for preventing infection and prevention of disease symptoms (*i.e.* control of infection) may be different [76]. In the case of preventative vaccines, an effective initial response to the virus is needed. At the time of the initial assault, the immune system is not dysfunctional. In contrast, during HIV therapy the immune system is in a state of dysregulation due to the constant tug of war with the virus infection [77-79]. Figure 8 and Table 1 below simplifies the current knowledge of immune dysfunction and pathogenesis of HIV infection [80]. Immune dysfunction has been identified in lymphocytes (T and B cells), NK cells, macrophages and increase or decrease in the levels cytokine secretion. The HIV immunotherapeutic field does not only have to establish the correlate for preventing disease progression, but has to overcome the immune dysfunction caused by the viral infection. The moderate success of the Thailand study and the failure of the STEP/ Merck Trial have brought into question both humoral and cellular immunity alone as the correlate(s) of protection. New vaccine designs are now aimed at inducing both humoral and cellular responses. The key to overcoming these obstacles faced by drug and vaccine development is continued research not only in terms of treatment, but also basic research of HIV and human immunology.

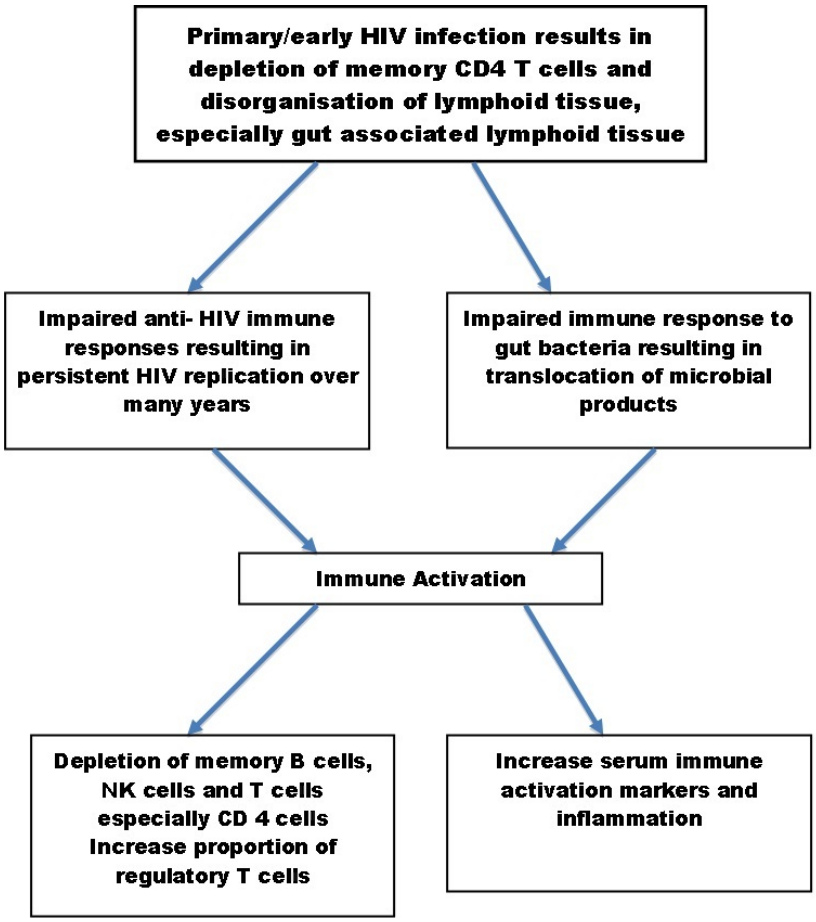

Figure 8: Pathogenesis of immune dysfunction associated with HIV

(Fernandez, S., A. Lim, et al. (2009) Journal of HIV Therapy 14(3): 52(55).)

The first phase occurs during acute infection and the early part of chronic infection where substantial depletion of memory T cells and disruption of the structure and function of the lymphoid tissues. This disruption of the immune lymphoid tissue set the stage for a state of persistent immune activation. This persistent immune activation state is characterized by increase serum immune makers and inflammation and depletion of memory cells (B cells, NK, and T cells).

Table 1: Evidence of dysfunction of the immune system of HIV infected individuals

Fernandez, S., A. Lim, et al. (2009) Journal of HIV Therapy 14(3): 52(55).

Dysfunction	References
The frequency of $CD4^+$ T cells infected by HIV in vivo is too low to account for the $CD4^+$ T cell loss	[81],[82]
Most apoptotic CD4+T cells in peripheral blood and lymph nodes of patients with chronic HIV infection are infected HIV	[83]
Naïve CD8+T cells, memory B and NK cells as well as CD4+T cells decline in HIV infection	[84],[85],
SIV-infected macaques exhibit a persistently activated immune system and rapidly progress to AIDS, while SIV-infected sooty mangabeys show normal T cell division rates and do not progress to AIDS.	[86]
HIV-2 infection is associated with lower levels of immune activation, which may explain the slower decline of CD4+Tcells compared with HIV-1 infection	[87]
In mice, TLR7 stimulation unrelated to a virus infection induces immune activation and immunopathology similar to that in HIV infection	[88]

1.4.2 HIV Vaccines

To date prophylactic vaccines available for infectious diseases have been based on the exploitation of the humoral immune response to the infectious organism for example influenza A, human papillomavirus, small pox. Therefore, it was logical to focus the initial HIV vaccine efforts on designing vaccines that induce protective humoral responses. The immunogen of choice was the envelope protein that is needed for viral entry into susceptible cells [89, 90]. Studies were done with the envelope proteins of both HIV and SIV in the non-human primate model. Some of the initial studies used either the envelope's soluble portion gp120 or the entire unit gp160 to induce a humoral response [91, 92]. HIV and the SIV envelopes were used for vaccination in the non-human primate model to test the proof of concept. These envelope subunit vaccines were shown to be immunogenic in both animals and in human trials and showed no toxicitiy in individuals. Also, the humoral response produced by vaccination by these subunit vaccines was capable of neutralizing lab-adapted viruses. With such promising results in preclinical and early clinical trials, the lack of vaccine efficacy in human trials was unexpected and very disappointing. The first phase III clinical trial in pursuit of an effective HIV vaccine was done by VaxGen using their vaccine called AIDSVAX and consisted of a bivalent subunit recombinant gp120 envelope, two clade B envelopes proteins [93].

After showing protection against homologous and heterologous challenge in a chimpanzee model, the VaxGen vaccine moved into clinical trials. The controversy was due to the fact that the primate study was not very well powered and complete protection was not achieved in a suboptimal HIV animal model [92]. The initial VaxGen phase I and phase II studies were done with a monovalent vaccine either from the clade B HIV strains MN or IIIB, both lab adapted strain viruses produced in engineered bacteria. In these early trials over 1200

individuals were enrolled with 600 being HIV negative and the rest being infected. Results from the phase I and II clinical trials showed that AIDSVAX was well tolerated however 6 individuals were found to acquire HIV during the trial. It was argued that the studies were not adequately powered to determine efficacy and in an effort to overcome this setback the vaccine that was sent into phase III clinical trial was bivalent with both strains MN and IIB. The vaccine trials took place in the US with a total of 5000 at risk women and homosexual men and in parallel in Thailand with intravenous drug users. The vaccines were tailored to the site such that in the US the vaccine consisted of envelopes from clade B and in Thailand the vaccine was made from envelopes from clade B and E. Trials were powered to determine efficacy and designed to be a three year study allowing for volunteer follow up. The endpoints designated for the trial were infection measured by seroconversion and viral load measured by polymerase chain reaction. In 2003, VaxGen reported the failure of its vaccine trial: there was no significant decrease in infection in individuals who received the vaccine when all individuals were considered. However, the company claimed that vaccine was more immunogenic and produced higher levels of antibody responses in Black and Asian volunteers. Because of this finding the AIDSVAX will be included in a future study in combination withALVAC-HIV-vCP1521 in Thailand. This trial will be covered later in this section [94].

Since the beginning of the VaxGen trials, HIV prophylaxis vaccines have increased in sophistication. Given the lack of evidence to support humoral responses protecting from infection, another correlate of protection was needed. Numerous studies in both animals and humans showed that $CD8^+$ T cells were linked to reduced viral load. These observations led vaccine development aimed at producing the ideal cellular responses which was not yet defined. In an effort to induce cellular immune responses, DNA vaccination and the viral vector-based

vaccines were adapted to the field of HIV. Viral vectors used for HIV vaccine development include poxvirus vectors, vaccinia virus vectors, adenovirus vectors, alphaviruses vectors, avipoxvirus vectors, poliovirus vectors and rhabdovirus vectors [95]. The use of viral vectors allow for high levels of antigen production at the site of vaccination. The viral nature and the particle characteristic of the vector system allows for efficient uptake of vaccine by professional antigen presenting cells to stimulate the immune response. In addition, viral vectors have mucosal adjuvant effects, which are an asset in HIV vaccine development, since most infection transmission takes place via the mucosa. However, there is a major disadvantage to viral vectors especially in the case of adenovirus: the possibility of preexisting immunity may lead to adverse effects and reduced immune response directed towards the vaccine antigens. The first HIV vaccine designed to produce a cellular immune response used an adenovirus vector to deliver its antigens. This trial was called the STEP trial and was headed by Merck. The ability of other strategies to elicit cellular response has been under investigation. One of the vaccine regimens that had proved successful in primates to produce a cellular response is a DNA prime followed by protein or viral vector boost[96, 97].

The Merck vaccine consisted of three recombinant adenoviral vectors expressing the genes *gag, pol and nef*. Pre-clinical trials in macaques vaccinated with the modified Adenovirus serotype 5 expressing the genes mentioned above showed immunogenicity and reduced viral loads when challenged with either SHIV virus (expressing an HIV envelope) or SIV_{mac239} virus. However, the animals' HLA played a role in the efficacy of vaccination [98]. Even with a less than ideal response in the primate model, as with the VaxGen vaccine, the vaccine was moved into clinical trials. During clinical pre-phase III trials should that the vaccine was safe and that it induced cellular immune via interferon gamma enzyme-linked immunospot assay (ELISPOT),

the standardized assay for cellular responses in a vaccine setting. STEP phase III trial enrolled a total of 3,000 healthy individuals. The endpoint for the STEP trial, as with the VaxGen study, was infection and viral load. Each volunteer received three injections of the three genes and received vaccination other two vaccinations two and three 6 months apart from each other. The STEP trial was stopped due to the results pointing to increased infectivity in the vaccinated groups. This outcome was unexpected as the volunteers in the vaccine arm of the study had quality cellular responses by ELISPOT and the CD8+ T cells generated were polyfunctional [99, 100], which was hypothesized to be needed for the ideal response to reduce viral load. However, the STEP results had an additional element which complicated the outcome: the rate of infectivity in volunteers who had a trend to higher preexisting immunity to Ad5 [75].

The failure of the STEP trial brought into scrutiny both the use of viral vectors and the standards that vaccines need to accomplish in animal models before moving into clinical trials. In both the VaxGen and Merck preclinical trial data showed limited success in NHP but were allowed to move into human trials. The urgency for an HIV vaccine, either preventative or therapeutic has led to vaccines without 100% protection in non-human primates moving into clinical trials. In the case of the viral vectors, overcoming the hurdle of preexisting immunity has involved modifying vector delivery and engineering vectors to prevent a reoccurrence of the Merck trial. For adenovirus vectors specifically, the following strategies have been proposed: a) immunosuppression of Ad responses by transiently depleted responses to the vector, b) create an artificial envelopes which results in a lack of recognition by the immune system, c) use alternative Ad serotypes which are rare in the human population, d) modify the vector to use alternative receptors thereby changing viral tropism and avoiding innate responses, e) use microspheres to encapsulate the vector and thereby by-passing the vector response and f) remove

early genes of the Ad and therefore there is not an initial response to the Ad vector [101]. Other viral vectors are also being engineered to deliver antigens and without stimulating an immune response to the vector itself. One such example is the engineering of a DNA launch platform for alphaviruses vector. This modification allows the alphavirus to be delivered as a DNA plasmid and therefore the structural proteins are not needed to enter cells to produce high amounts of antigens [102].

After the failure of the STEP vaccine trial, three different views emerged on the possible correlate of protection from HIV transmission: 1) humoral/neutralizing antibodies, 2) cellular or CD8 T cells and 3) a balance of humoral and cellular responses, the results of the next phase III vaccine trial were of great interest. The ALVAC and AIDSVAX clinical trial in Thailand enrolled 16402 healthy men and women between the ages of 18-30 years into the study [69]. The ALVAC vaccine contains a clade E *envelope* with a *gag/pol* from clade B and the AIDSVAX vaccine is the B/E vaccine covered in the previous section. The vaccine trial covered multiple centers and the individuals that were randomized into placebo or vaccine groups. Vaccine groups received four injection of the canarypox virus vector vaccine ALVAC, followed by two booster injections with the AIDSVAX B/E recombinant gp120. The endpoints for the trial were HIV infection and early viremia after the first 6 months and every 6 months thereafter for 3 years. Measurement of cellular immunogenicity was done by interferon gamma ELISPOT and intracellular cytokine staining following stimulation with antigens gag and envelope [103]. Humoral responses were measured for binding antibodies to various gp120 envelopes and p24 (gag core). T cell responses via ELISPOT showed a 19.7% induction in vaccinated individuals 6 months after the final vaccination. In addition, greater cytokine responses were measured in the CD4+ T cells of vaccinated individuals. Binding antibodies to the envelopes MN and A244

present in the vaccine were similar and had a geometric mean titer T-1 of 31,207 and 14588 respectively. There were only mild to moderate adverse effects mainly at the site of injection, confirming findings in preliminary studies. There was no significant difference in individuals who became infected whether or not they received the vaccine. Nonetheless, there the study recorded a 31.3% protection rate using a 95% confidence interval. Even with the moderate outcome, this was the first time any efficacy was reported in an HIV vaccine trial. In addition to being of such low efficacy, there was still no identified correlate of protection. The only parameter that showed any potential as a correlate of protection was antibody binding to envelopes. This vaccine trial infused a new hope into the HIV vaccine field, showing that protection from infection was possible.

Some of these challenges include vaccine design to overcome variability and inducing the appropriate immune response at the mucosal surface. Strategies being used to overcome virus variability are using centralized sequences, usually based on envelopes of one or multiple clades, and polyvalent vaccines consisting of multiple genes of HIV from one or multiple clades [104] [105]. To induce mucosal immunity different vaccine strategies including vaccination at the mucosa (oral or vaginal in primates) and use of adjuvants are being used. However, this task is difficult one since the immune environment during infection and what is needed to prevent infection is not well understood or known.

1.4.3 Animal Models

Another hurdle in the field is the use of an appropriate animal model for testing immunogenicity and efficacy of the vaccines. To date, macaques infected with SIV or SHIV are the most widely NHP as an animal model in AIDS research. Other models that have been used, include

chimpanzees infected with HIV-1, cats infected with feline immunodeficiency virus (FIV), rodents including humanized severe combined immunodeficiency mice (SCID/hu mice), and guinea pigs and rabbits mainly used for immunogenicity of vaccines [106, 107]. Each animal model has its pros and cons. In the case of chimpanzees, while these animals can be infected with HIV-1 the replication of the virus is not as robust as seen in humans and they do not progress to any immunodeficiency disease. However, vaccine efficacy may be valuable since there can be a direct challenge of HIV and not SIV or SIV/HIV chimera [92]. The other drawback to chimpanzees is the cost of the model since they are an endangered species. Due to their protective status after a study is done these animals are required to have post trial care which requires a huge budget. Such cost limits the number of animals afforded for a study thereby diminishing any chance for statistical significance and usually does not justify its use.

Two less expensive models in which the appropriate numbers of animals needed to reach statistical significance can be achieved are FIV in cats and rodents. FIV in cats is a good model because it is a naturally occurring lentivirus infection and disease. During FIV infection, cats also experience CD4+ T cell depletion [108, 109]. The FIV model has been used to better understand transmission and immunopathogenesis. Given these characteristics, this model provides a less expensive option for beginning *in vivo* studies of antiviral drugs and also emergence of resistance to antiviral drugs [110]. One of the more poorly understood aspects of HIV is transmission and mucosal immune responses. Human studies are limited for analysis of mucosal immunity and research lies heavily on animal models, hence one of the areas of interest in the FIV /cat model. Although the feline model is attractive, the viruses HIV and FIV are significantly genetically different and the immune system of cats does not mimic that of humans [111]. Additionally, an FIV infection results in the infection of not only CD4+ T cells but also

CD8+T cells and B cells [112]. This phenomenon of infection of multiple types of lymphocyte cells is not seen during HIV infection.

One of the major benefits of rodents in science is the inexpensive, reduced space needed for housing and the ease of repeating experiments (e.g. BaLB/c). For this reason, many preliminary experiments evaluating vaccine immunogenicity are usually performed in mice. However, the drawback of the rodents is the inability to be infected with HIV, which has limited its use as a model. To overcome the inability of HIV to infect mice, the SCID/hu mouse was developed. This model allows for the dissection of individual components of a human response to vaccines and its impact on HIV infection [113]. Nonetheless, HIV virus replication in this model, like the chimpanzee, is limited and the humanized mice do not mimic the pathogenesis of developing immunodeficiency. Despite these limitations, HIV dissemination can be evaluated in this model [114]. Over the years this SCID/hu model has evolved from just reconstituting immune cells to completely reconstituting all hematopoietic lineages by lymphoid organs such as bone marrow, liver and thymus of human origin [115].

NHPs most often used for HIV/SIV studies are rhesus macaques, cynomologous macaques and pigtail macaques. The NHP macaque SIV infection model allows for insight into initial infection and pathogenesis with the added advantage that SIV shares the same receptor as HIV: CD4. In addition, SIV and HIV are genetically similar and SIV can be modified to allow investigation of certain proteins to investigate their specific role in infection and/or pathogenesis [116]. The macaque model can also be infected with HIV-2, but not HIV-1, to overcome the dilemma of chimeric viruses when SIV genes are replaced with the gene of interest from HIV are used. Additionally, the pathogenesis of NHP SIV infected model, is similar to the pathogenesis of HIV-1 in humans and not only allows for prophylactic drug testing, but also drug therapy for

viral infection and disease [117]. Furthermore, the macaque model has been used to investigate infection using multiple routes of infection: oral, intravenous, intra-vaginal, and intra-rectal thereby mimicking human transmission.

Extensive use of the NHP model has resulted in the ability to mimic different outcomes of infection as is seen in humans. The outcomes of infection and disease progression are dependent on host selection and the SIV clone chosen for infection. The different outcomes of disease are similar to those observed in HIV human infection: from high viremia to control of viremia and slower disease progression to rapid progression to disease. Multiple NHP have been infected by different SIV and the infection and disease progression are being studied in order to identify elements that can aid in development of new treatments and/or preventative vaccines [118-120]. The field of animal AIDS modeling has also gone on to model and compare juvenile and adult infection, vertical transmission from mother to offspring and expanded the species of NHP used to include African Greens. While the NHP model provides a chance to collect an immense amount of knowledge, an important caveat is that the model remains a surrogate of the actual infection being studied and there are limitations. The major limitation of the NHP model is the fact that HIV does not infect most monkeys except for gibbon apes and chimpanzees [121]. However, infection of gibbon apes and chimpanzees is usually not associated with clinical disease and haematological abnormailities [107, 121, 122]. Other differences between humans and NHP that may be relevant to the disease include the difference in composition of bacterial flora in the gut [123], genome differences and the biology at subcutaneous vaccination sites [107]. Differences in bacterial flora may influence mucosal immunology patterns that may affect mucosal vaccine responses especially if administered orally. Mucosal response to vaccination is important in HIV vaccination since most infection occur via a mucosal route. A study by Gibbs

et al. identified that there were more active immune genes in rhesus macaques genome than in humans [124]. These genome differences support the need for cautious interpretation of data collected from rhesus macaque model. Differences in composition and distribution of muscle fibers, prevalence of interstitial and intra-tissue fat and tissue vascularization can directly influence vaccination distribution, diffusion of the formulation, its rate of clearance and the types of host cell encountered. These differences may contribute to differences in immune responses induced by vaccination.

1.4.4 Methods used to overcome Diversity

1.4.4.1 Centralized Vaccine

The aim of centralized vaccines in HIV vaccine development is to overcome the high diversity of the virus. The three main methods for developing centralized vaccines are consensus, center of tree and ancestral [125-129]. These vaccines are made in an effort to reduce the differences between the vaccine antigen and circulating viruses while maximizing the breadth of reactivity coverage of the immune response. The consensus method is more likely to have epitopes relevant to current epidemic strains than either ancestral or center of the tree [126]. The ancestral sequences are predicted based on older, non-contemporary sequences. Consensus vaccines based on various HIV immunogens including Gag and Env have been studied [130, 131]. Over 95% of the global epidemic is due to HIV-1 group M viruses. Therefore, most of the consensus vaccines being studied are based on clades within group M or on Group M as a whole. These consensus vaccines are antigenic and immunogenic and elicit stronger and more diverse immune responses than vaccines based on primary sequences [132, 133]. Consensus vaccine

studies elicit in cross-clade neutralizing antibodies [133-135]. Consensus HIV-1 Env vaccines have also elicited broad cellular and humoral immunity in NHPs [136].

1.4.4.2 Polyvalent Vaccines

Like centralized vaccines, polyvalent vaccines are used to increase the breadth and strength of immune responses elicited by a given vaccine [137]. Unlike centralized vaccines polyvalent vaccines are made up of multiple antigens that may consist of a variation of the same antigen (multiple primary Envs from different strains) or multiple antigens found in the pathogen (Env, Gag, Pol). These vaccines have been tested in preclinical and clinical trials where they are safe and provide some level of protection [138]. In polyvalent vaccine studies in mice and primates, there has been evidence of broadly reactive humoral and cellular immune responses. A human clinical trial by Wang *et al*. showed that a polyvalent DNA prime–viral vector boost vaccination results in cross-subtype antibody and cellular immune responses [139]. Polyvalent vaccines have also elicited protection and viremia control in preclinical NHP trial studies [140]. Polyvalent vaccines have been successful in against other diseases such as influenza and human papillomaviruses *(*HPV).

1.5 ADJUVANTS

The first vaccines used in humans were either live attenuated or whole killed organisms that induced long lasting immunity with little or no booster shots to maintain immunity. However these vaccines are reactogenic, induce mild disease and have the possibility of reverting to virulence [141]. Live attenuated and whole killed vaccines are not suitable for

immunocompromised individuals. Also, the possibility of reversion and contamination of live organisms in killed bacteria preparations make this vaccine method too risky in the case of diseases such as HIV. Since Jenner's first vaccination experiment with cowpox, a better understanding of the immune response to pathogens and improve technology has led to safer subunit vaccines (protective antigen of an organism e.g. bacterial toxin). These subunit vaccines are less immunogenic than attenuated or killed vaccines and require multiple booster shots. Early studies by investigators such as Glenny and colleagues showed proof of concept that immune responses elicited by subunit vaccines can be improved using substances termed adjuvants [142]. Glenny reported a vast increase in anti-toxin antibodies in guinea pigs, when the diphtheria toxoid vaccine was with alum versus untreated toxoid. An immunological adjuvant can be defined as a substance that works to accelerate, prolong and /or enhance an antigenic–specific immune response when combined with a purified antigen [141, 143-145].

1.5.1 Adjuvants mechanism of action

Adjuvants work in a variety of ways. The mechanisms of action can be classified into five main categories: i) antigen clustering, ii) size of vaccine particles, iii) antigen depot, iv) targeting vaccines to particular cells and v) stimulating the innate immune pathways [141].

i) Antigen clustering: Most pathogens have surfaces consisting of repeated structures such as lipopolysaccharide (LPS) on gram negative bacteria. These high order structures are detected by the immune system enhancing antigen uptake and recognition and activation of B cells. Pentraxins, natural IgM, mannose binding lectin, C-type lectin all recognize highly repetitive antigens. Clustered antigens had been shown to efficiently activate B cells through their

surface immunoglobulin receptor resulting in increased proliferation and differentiation and subsequently higher titers [146]. Order clustered arrays of a component of the cell wall of yeast and fungi beta-glucan was shown to activate dectin-2 signaling when presented as a particulate coating [147, 148]. The beta-glucan coating triggered phagocytosis and immune activation. Ordered particulate surfaces are also bound by IgM and complement components allowing more efficient uptake by lymph node macrophages which transfer the antigens to follicular dendritic cells. The dendritic cells maintain the antigens to allow long-term presentation to B cells in the lymph node leading to increase immunity [149].

ii) The size of the vaccine particle affects its cellular uptake and migration patterns after administration. Particle sizes of ~40nm are more efficiently taken up by dendritic cells [150, 151] and more likely to induce interferon gamma (vital in innate and adaptive viral and intracellular pathogens) [151]. While larger particles (>200nm) need to be transported from the injection site to the lymph nodes by monocytes, neutrophils and dendritic cells and induce more IL-4 from helper T cells (Th2: humoral immunity) [151-153]. Having this knowledge and vaccine delivery tools such as liposomes and polymers multiple vaccine vehicles can be tested.

iii) Maintaining and antigen depot: One of the drawbacks to non-replicative vaccines is the need for repeated vaccinations due to the short-life of the antigen. Follicular dendritic cells are known to maintain antigens in the lymph nodes allowing for interaction with antigen specific B and or T cells. If the same

principle is applied to vaccines, persistent antigen presentation would allow for reduce number of required vaccinations for prolonged immunity. Examples of methods used to contain antigens are micro-particles and slow release implants [154]. The use of antigen depot methods results in slow release of the antigen overtime and protects the antigen from quick degradation allowing for persistent immune stimulation [155].

iv) Targeting to specific cells: Antigens are fused with antibodies or triggering molecules that bind specific cell receptors (*e.g.* C3d targets B cells) [156, 157]. Cell types that are usually targeted are dendritic cells [158, 159] because of the most effective antigen presenting cells and B cells because antigen specific antibodies are the goal of the vaccine administered. Antibodies can neutralize toxins, inhibit cell invasion, or work with T cells in antibody mediated cellular cytotoxicity.

v) Stimulating the innate immune pathways: The innate system does not only alert the adaptive system of a pathogen but also dictates the response to a particular infectious agent. The discovery of pathogen-associated molecular patterns (PAMPS), pattern recognition receptors (PRRs) and C-type lectin receptors has shown the importance of the innate system in modulating the response to immune invaders. Innate immune receptors dictate the expression and activation of distinct pathways in the cell leading to induction of immune modulating molecules such as cytokines, chemokines, leukotrienes and prostaglandins. Table 1 has a list of currently identified innate receptors, the ligands, location and outcomes upon activation. New vaccine production and purification techniques

removed most of the ligands required to activate these innate ligands (*e.g.* LPS and viral RNA genomes). Hence the use of adjuvants formulations with vaccines such as toll-like-receptor (TLR) agonist allows for manipulation of these innate pathways to elicit an effective immune response to a specific antigen. TLR ligand monophosphoryl lipid A (MPL) is formulated with alum in the FDA approved HPV vaccine and increases the immunogenicity of the vaccine reducing the number of doses required for a sufficient antibody production [160].

1.5.2 Types of adjuvants

Adjuvants can be divided into main categories based on the era of discovery: i) classical and II) novel adjuvants. The classical adjuvants include aluminum salts, emulsions and liposomes and virosomes. The novel adjuvants include TLR agonists, Saponins, Immune stimulating complexes and adjuvants systems.

1.5.2.1 Classical Adjuvants

a) Aluminum salts (alum): Alum was the first licensed adjuvant [161, 162]. The first use of aluminum salts was with diphtheria toxin and it was assumed that alum worked as an antigen depot [142]; since then that theory has been disproven. Recent studies have shown that alum allow antigens to be presented in a particulate form allowing more efficient internalization of the antigens by antigen presenting cells (APC) [163]. Additionally alum has been shown to activate components of the inflammasome complex (member of the NOD-like family of PRRs) resulting in the release of proinflammatory cytokines such as IL-4, IL-5, IL-1β and IL-18 [164, 165]. Alum is

also associated with a Th2 or humoral responses. Alum is used in a number of vaccines, including vaccines for hepatitis A and B, HPV, *Streptococus pneumonia*, pediatric diphtheria, tetanus and pertussis.

b) Emulsions: Emulsions are of two main categories either water in oil or oil-in-water emulsion. The first of these adjuvants was the water in oil emulsion incomplete or complete Freund's adjuvant. Freund's adjuvant was too reactogenic for use in humans and were discontinued [141]. Since Freud adjuvant new water-in-oil emulsion such as Montanide emulsion are being tested for therapeutic vaccines in clinical trial [166]. Oil-in-water emulsions are postulated to involve innate inflammatory responses, APC recruitment and activation, elicitation of cytokines and antigen persistence at the site of vaccination [167]. The oil-in water emulsions have been successful in human trials and licensed vaccines. MF59 an example of an oil-in water emulsion adjuvants became a licensed adjuvants used in the seasonal influenza vaccine *Fluad*™ in humans [168]. Clinical trials with MF59 have resulted in enhanced humoral and cellular responses to pathogens such as HIV, Influenza, and HSV [169].

c) Liposomes and Virosomes: Liposomes are synthetic lipid layer nanospheres used to encapsulate antigens [170]. Virosomes are empty envelopes of the influenza virus. Both liposomes and virosomes act as vaccine delivery systems [171, 172], with virosomes having the additional advantage of having a viral envelope aiding in cell uptake and membrane fusion to aid in antigen delivery [173]. Virosomes have been used in licensed vaccines such as *Inflexal*™, and *Epaxal* ™. The *Inflexal*™ influenza vaccine is immunogenic in all age groups, even in the immunocompromise elderly [174, 175].

1.5.2.2 Novel Adjuvants

a) Toll-like-receptor agonists (TLR): These adjuvants are an example of immunoenhancers. Immunoenhancers are adjuvants that work by direct stimulation of the innate system (Table 2) [176]. They act on pathogen-associated molecular patterns such as TLR on innate cells such as monocytes, macrophages, and dendritic cells. TLR agonists include: 1) MPL (safe formulation of LPS) interacts with TLR4, 2) immunostimulatory sequences of microbial DNA eg. CpG oligodeoxynucelotides (CpG ODNs) interacts with TLR9, 3) Imidazoquinolines (eg. Imiquimod, Resiquimod) interacts with TLR7 and TLR8. MPL has shown to stimulate the expression of co-stimulatory molecules and cytokine release improving the both the quantity and quality of humoral and cellular immune responses depending on the antigen [177-179]. CpG ODNs induce inflammatory cytokines such as TNF-α, IL-1, IL-6, IFN-α, and IFN-γ and have shown to induce Th1 immunity (cellular responses) and cytotoxic T-lymphocyte responses [180]. Imidazoquinolines induce the same immune response as RNA binding to TLR [181]. Imiquimod formulated with a prototype vaccine has demonstrated extend both the duration and the extent of protection against HSV in mice [182].

b) Saponins: These substances are immunoenhancers derived from plants. Quil A (QS21) is derived from the back of a tree called *Quillaja saponaria* [183, 184]. QS21 enhance antigen presentation to APCs and predominantly induces cytotoxic lymphocyte and Th1 and Th2 cytokine secretion upon animal vaccination [185, 186]. Saponins are now being formulated into the latest novel adjuvants called adjuvants systems [183].

c) Immune stimulating complexes (ISCOMs): ISCOMs are cage-like complexes composed of lipids, cholesterol, antigen, and Quil A. The hydrophobic interactions of the ISCOM trap the antigen and allows for more efficient endocytosis by APCs. These adjuvants has resulted in enhanced humoral and cellular responses in animal models [187]. ISCOMs have also been modified to exclude antigens form the formula called ISCOMATRIX, allowing the adjuvant to be used to any antigen [188]. ISCOMATRIX presents antigens by both MHC class I and II pathways [189]. In addition ISCOMATRIX increases the longevity and magnitude of humoral immune response [189-191]. Currently these adjuvants are being investigated for Influenza, HCV, and cancer [192-194].

d) Adjuvant Systems: The adjuvant systems combine the characteristics of both classical and novel adjuvants. These systems allow the immune response elicited by vaccine antigens to be enhanced in more than one adjuvant. The combination of more than one adjuvant allows for activation and modulation of the immune system at more than one level [195, 196]. Some adjuvant systems include MPL and QS21 (novel adjuvants) combined with aluminum salts, liposomes and or oil-in water emulsions (classical adjuvants) to create adjuvants systems AS04, AS02, AS01 and AS05. Table 3 describes the components of multiple adjuvant system being investigated by GSK Biologicals.

Table 2: Characteristics of currently identified innate immune receptors and their legends

Mike de Veer ME. Discov Med 2011;12(64):195-204

Defined Ligand	Receptor	Location	Outcome	Reference
Nucleic Acids:				
DNA	HIN200, AIM2, DAI	Cytoplasmic	Interferon	Roberts et al., 2009; Takaoka et al., 2007
Nonmethylated CpG DNA	TLR9	Endosome	Th1, CTLs	Klinman, 2006
ssRNA	TLR7/8 IFIT (5'triphosphate)	Endosome Cytoplasmic	Interferon, Th1 Interferon	Pichlmair et al., 2011
dsRNA	Rig-I (5'triphosphate) MDA5 (long) TLR3	Cytoplasmic Cytoplasmic Endosome	Interferon Interferon Interferon	Alexopoulou et al., 2001; Kato et al., 2008
Imidazolquinolines (Imiquimod, R-848)	TLR7	Endosome	Interferon, Th1	Hemmi et al., 2002
Amidinothiazoline (3M-002)	TLR8	Endosome	Interferon, Th1	Philbin and Levy, 2007
Pathogen PAMPs:				
Peptidoglycans Des/muramyldipeptide (DMP and MDP)	TLR2, NOD1, and NOD2	Plasma membrane, cytoplasmic	Th1, CTLs	Ozinsky et al., 2000; Uehara et al., 2006
Lipopeptides (PAM-3-Cys, MALP2)	TLR2+1 or TLR2+6	Plasma membrane	Th1, CTLs	Duthie et al., 2011
Lipopolysaccharides (LPS, MPL)	TLR4	Plasma membrane	Th1, CTLs	Duthie et al., 2011
Lipoteichoic acid (LTA)	TLR2	Plasma membrane	Th1, CTLs	Duthie et al., 2011
Flagellin	TLR5, NLRC4	Plasma membrane, cytoplasmic	Th1, CTLs	Franchi et al., 2006; Hayashi et al., 2001
Secretion system rod protein	NLRC4	Cytoplasmic	Th1, CTLs	Miao and Warren, 2010; Muller et al., 2009
Beta Glucan	Dectin 1 (CLEC7a), TLR2	Plasma membrane	Th17 cells	Smeekens et al., 2011
Alpha-mannans	Dectin 2	Plasma membrane	Th1, Th17	Drummond et al., 2011; Saijo et al., 2010
Alpha-mannose (Fungi)	Mincle	Plasma membrane	Th1, Th17	Drummond et al., 2011
Trehalose-6,6-dibehenate (TDB)	Mincle	Plasma membrane	Th1, Th17	Marakalala et al., 2011; Schoenen et al., 2010
Trehalose-6,6-dimycolate (TDM)	Mincle	Plasma membrane	Th1, Th17	Ishikawa et al., 2009; Marakalala et al., 2011
Lipoarabinomannan	TLR2	Plasma membrane	Th1, CTLs	Underhill et al., 1999
Mannose, fucose	MBL MMR	Plasma membrane Plasma membrane	Phagocytosis Complement	Lee et al., 2011; Taylor et al., 2005
Toxins:				
Anthrax lethal toxin	NLRP1	Cytoplasmic		Newman et al., 2010
Complement:				
Complement: C3d, C5d	CR2	Extracellular	Phagocytosis, Antibody	Dempsey et al., 1996
Allergens:				
Allergen glycans/HDM	Dectin 2	Plasma membrane	Th2	Barrett et al., 2011
Others:				
Advanced glycation products, fibrillar proteins, oxLDL	RAGE	Plasma membrane	Inflammation	Yan et al., 2010
Aluminium adjuvants	NALP3	Cytoplasmic	Th2, Antibodies	Hornung et al., 2008
Saponins	NLRP3	Cytoplasmic	Th1, CTL	Bauernfeind et al., 2011; Duewell et al., 2011

Abbreviations: TLR, Toll like receptor; Th, T helper; NOD, Nod like receptor; NLRP, Nod like receptor protein; IFIT, interferon-induced protein with tetratricopeptide repeats; MDA, melanoma differentiation associated; MMR, macrophage mannose receptor; RAGE, receptor for advanced glycation endproducts; oxLDL, oxidized low density lipoprotein; CTL, cytotoxic T cells; CR, complement receptor; HDM, house dust mite; DC, dendritic cell.

Table 3: GSK Biologicals Adjuvant System components and applications in the late stage of clinical development

Leroux-Roels G. Vaccine 2010;28, Supplement 3(0):C25-C36

Adjuvant system	Components	Current applications
AS01	MPL, QS21, liposome-based	Malaria, tuberculosis
AS03	α-Tocopherol oil-in-water emulsion-based	H5N1 influenza, seasonal influenza, H1N1v influenza (*Arepanrix™*, *Pandemrix™*)
AS04	MPL absorbed on aluminium salt	Hepatitis B (*FENDrix™)*, HPV (*Cervarix*™), *Herpes simplex* virus (HSV) vaccine,
AS15	MPL, QS21, CpG, liposome-based	cancer immunotherapy, Non-Small Cell Lung Cancer (NSCLC), melanoma

1.6 $SHIV_{SF162P4}$ CHALLENGE

The discovery of a monkey lentivirus which caused and AIDS-like disease following inoculation in an Asian macaque has now lead to the use of nonhuman primates as the preferred model for study HIV vaccines. This HIV related virus is called simian immunodeficiency virus (SIV). The SIV genome organization is similar to that of HIV with the *vpu* of HIV absent and the addition of

the gene *vpx*. The genomic similarities of SIV and HIV do not allow infection of nonhuman primates with HIV except in the case of chimpanzees. Therefore, a strategy needed to be developed to investigate HIV genes and proteins in the non-human primate model. To overcome this obstacle the idea of constructing an SIV/HIV chimera (SHIV) for infecting non-human primates was established. Multiple SHIVs with HIV genes such as *tat, rev, env* and portions of *nef* have been constructed and tested [197]. The SHIV virus used in this project was chosen based on its use of CCR5 use as co-receptor for infection, CCR5 has been identified as the co-receptor used by transmissible viruses during infection. In addition the SHIV chosen has the ability to infect at the mucosal surface (vaginal, and rectal). Contribution of HIV mucosal sites (vaginal, rectal and intestinal) to the global HIV infection is estimated to more than 47.5 million infections [198]. For this study, the route of virus inoculation will be rectal. Furthermore, the SHIVs allow for the investigation of HIV proteins example HIV Env in a non-human primate model.

2.0 MATERIALS AND METHODS

2.1 VACCINE CONSTRUCTION AND CHARACTERIZATION

2.1.1 Consensus envelope Design

One hundred sequences per clade were used to design consensus sequences of clades A, B, C, and E. These sequences were obtained from multiple sources (LANL.B. Hahn-UAB, C. Williamson-South Africa, P. Gupta-India, Y. Shao-China, D. Montefiori-Duke, D. Ellenberger-CDC, G. Nabel-NIH, R. Center-Australia and the AARRP). The design of the group M consensus envelope was based on two hundred Env sequences representing clades A, B, C, D, E, F and H. The use of the CCR5 co-receptor was identified for 75% of the Envs. Each consensus Env_{gp160} was truncated at amino acid (aa) sequence WYIK (consensus M aa 665) to Env_{gp140} and the cleavage gp120/gp41 site was mutated by replacing the arginines (R) in the cleavage site with serines (S). Finally, the bacteriophage fibronectin (FT) domain was inserted at the 3' end of the Env sequence to stabilize the expressed Env protein as a trimer [1] Each consensus Env gene sequence was synthesized (GeneArt, Regensburg, Germany), cloned into the previously described pTR600 [199].

Envs were expressed from a cytomegalovirus immediate-early promoter (CMV-IE) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation

signal (BGH poly A) for termination of transcription. Each plasmid was amplified in *Escherichia coli* strain-DH5 alpha, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at -20^{o}C in dH_2O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at a wavelength of 260 and 280 nm.

2.1.2 Protein Purification

Human embryonic kidney (HEK) 293T cells were transiently transfected with 8µg DNA plasmid expressing one of the consensus HIV-1 Env_{gp140} proteins (Consensus A,B,C,E and SF162,YU2,ADA,R2,PVO4,SC42) and Env $_{gp120}$ histag proteins (SF162, CHN19, EIIF22070, Q23, Q259). Following DNA transfection using Lipofectamine2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), cells were incubated for 72 h in opti-MEM I reduced serum formula media (Gibco, Grand Island, NY, USA). Media was collected and secreted Env_{gp140} proteins were purified using lectin columns made from agarose galanthus nivalis (snowdrop) lectin (Vector laboratories, Burlingame, CA, USA). Env_{gp120} were purified at 4 ◦C using a 5 ml HiTrap chelating nickel column (Amersham Biosciences, Piscataway, NJ, USA). Briefly, the column was loaded with 0.1M NiSO4 (5 ml) (Fisher Scientific, Fair Lawn, NJ, USA) and subsequently washed with distilled water (15 ml). The HiTrap column was equilibrated with 30 ml of Binding Buffer (20mM phosphate, 0.5M NaCl and 10mM imidazole) (Sigma, St. Louis, MO, USA)]. Supernatants containing His-tagged proteins were loaded into the equilibrated HiTrap column at a rate of 2ml/min. The column was then washed with Binding Buffer (30 ml). Subsequently, the proteins were eluted using 15 ml of Elution Buffer (20mM phosphate, 0.5M NaCl and 500mM imidazole). Eluted proteins were dialyzed in PBS using Slide–A-Lyzer

dialysis cassettes (Thermo Scientific, Waltham, Massachusetts, USA). Proteins were then concentrated using Amicon ultra centrifugal filter concentrators (Millipore, Billerica, Massachusetts, USA). Micro BCA Protein Assay kit (Thermo Scientific, Waltham, Massachusetts, USA) was used to quantify proteins. Other purified envelopes used for ELISAs (PVO4, SC42, 6535, THRO4, AC10, RHPA, CAP210, ZM214, DU172) were purchased from eEnzyme (Gaitherburgs, MD, USA).

1ug of each purified consensus gp140 trimer protein was loaded unto NativePAGE native gel (Invitrogen Life Technologies, Carlsbad, CA, USA) and separated by electrophoresis in the manufacture's recommended buffers (NativePAGE running and anode buffers). After separation gel was silver stain using the ProteoSilver Sliver Stain kit (Sigma, St.Louis, MO, USA) following manufacture's protocol. Briefly, after electrophoresis the gel was placed in a clean tray with 100 ml of Fixing Solution (50% ethanol, 10% acetic acid in ultrapure water) for 1 h. The Fixing Solution was removed and the gel was washed with 100 ml of Ethanol Solution (30% ethanol in ultrapure water) for 10 min. The Ethanol Solution was decanted and the gel was washed twice (10 min) with 200 ml of ultrapure water. The gel was then incubated for 10 min with 100 ml of Sensitization Solution (1% ProteoSilver Sensitizer in ultrapure water). Following removal of the sensitizing solution, the gel was washed twice (10 min) with 200 ml of ultrapure water. The water was decanted and 100 ml of Silver Equilibration Solution (1% ProteoSilver Silver solution in ultrapure water) was added to the gel for 10 min. After the Silver Equilibration Solution was removed, the gel was washed for 1min with 200 ml of ultrapure water. The water was decanted and 100 ml of Developer Solution (5% of Proteo Silver Developer 1, 0.1% of ProteoSilver Developer 2 in ultrapure water) were added to the gel. The gel was carefully rocked

back and forth for 3–7 minutes until the desired staining intensity was observed. 5ml of the ProteoSilver Stop Solution was then added to stop the reaction.

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2.1.3 Virus- Like Particle (VLP) Production

Supernatants from COS cells (monkey kidney cells) transiently transfected with plasmids expressing either SIV Gag or consensus VLP were pelleted by ultracentrifugation (100,000xg through 20% glycerol, weight per volume) for 4 h at 4°C. Pellets were resuspended in PBS and stored at -20°C until needed. Protein concentration was determined using Micro BCA TM Protein Assay Reagent Kit (Pierce Biotechnology, Rockford ,IL. USA).

2.1.4 CD4 Binding Assay

Protein G Dynabeads (Invitrogen) were mixed to ensure homogenous suspension. 20ul of beads suspension was used per reaction. Magnetic beads were washed 3x using 500ul of citrate-phosphate buffer pH 5. Then 4µg of mouse anti-his antibody (Invitrogen Life Technologies, Carlsbad, CA, USA) was diluted in 35ul of citrate-phosphate buffer and was added to beads. The suspension was mixed at RT for 40 min. The tubes were then placed on the magnets to remove all unbound antibody and washed with citrate buffer 3X. Then soluble human CD4 HIStag (eEnzyme, Gaithersburg, MD, USA)/consensus envelope mixtures were then placed in with the beads and allowed to incubate for 1 hr at 4°C. Prior to this step the human soluble CD4 protein and consensus envelopes were at 37°C for 1 hr before being added to the beads. After 1 hr the

suspension was placed on a magnet and the liquid or supernatant fraction was collected. Beads or pellet fractions were then washed 3X with phosphate buffer saline (PBS) and then re-suspended in 50ul of PBS. Then samples were separated on a 10% SDS PAGE gel, transferred unto a nitrocellulose membrane and probed for sCD4 or envelope using mouse anti-human CD4 at 1:5,000 (Southern Biotech, Birmingham, AL, USA) and rabbit anti-IIIB gp120 envelope at 1:5,000 (Advanced Biotechnologies Inc, Columbia, MD, USA) respectively. Secondary antibodies goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were used at 1:10,000 (Southern Biotech, Birmingham, AL, USA). Western blots were developed using Pierce ECL western blotting substrate (Thermo Scientific, Waltham, Massachusetts, USA).

2.1.5 Biacore

Kienetic analyses of MAb b12 binding to HIV rgp140(s) was performed on a Biacore 3000 (GE/Biacore AB, Inc., Uppsala, Sweden). Protein A (Pierce, Rockford, IL) was covalently bound to individual flow cell surfaces of a CM5 sensor chip utilizing amine-coupling chemistry. MAb were captured and oriented onto protein A surfaces to ensure that the MAb-envelope binding occurred as a homogenous 1:1 Langmuir interaction. Three-fold serial dilutions of HIV rgp140(s) trimers were injected over each flow cell at concentrations ranging from 0.815nM to 66nM. The reference surface contained protein A that served to account for changes in the buffer refractive index and to test for potential nonspecific interactions between rgp140 and protein A. All runs were double referenced with a buffer alone injection serving as a negative control. Upon completion of each association and dissociation cycle, surfaces were pulsed with regeneration solution. Rhesus MAb association rates (ka), dissociation rates (kd), and affinity constants (KD) were calculated with BIA evaluation 4.1.1 software (GE/Biacore AB, Uppsala, Sweden). The

goodness of each fit was based on the agreement between experimental data and the calculated fits, where the 2 values were below 1.0. Protein A-MAb surface densities were optimized to minimize mass transfer, and kinetic analyses demonstrated that the binding interactions were not significantly mass transfer limited.

2.2 ANIMAL VACCINATION AND IMMUNE RESPONSE ANALYSIS

2.2.1 Animal Vaccination

Balb/c mice (*Mus Musculus* (6-8 weeks old) were used for the initial animal characterization experiments. These animals were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) and housed in microisolator units with access to free food and water. All vaccinations were given in the quadriceps and formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) in a 50µl total volume. Mice were vaccinated with 10ug or 20ug VLP of protein at weeks 0, 3 in the initial study and weeks 0, 4 the second mice experiments. In both studies mice were vaccinated in the quadriceps. Each mouse group consisted of ten (10) animals.

Rhesus macaques (*Macaca mulatta)* were used for all non-human primate experiments. All animals were cared for adhering to USDA guidelines for laboratory animals. Rhesus macaques were anesthetized using 10-20mg/kg ketamine and vaccinated intramuscularly in the quadriceps and formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) in a 1ml total volume. Vaccinations were completed at weeks 0, 4 and 8.

Twelve animals were divided into three (3) groups, four animals per group. Group 1 or Mock animals received adjuvant only. Group 2 or Polyvalent Consensus animals received a total of 300ug of total purified protein per vaccination (equal amounts of Consensus A, B, C, E envelopes). Group 3 or Consensus M animals received a total of 300ug total protein per vaccination (Table 4).

2.2.2 Sample Collection and Processing

For blood sample collection animals were anesthetized with a mixture of ketamine/xylazine. Anesthetized mice were bled at weeks 4 and 6 via the retro-orbital plexus, and blood was transferred to a microcentrifuge tube. Tubes were centrifuged and sera were collected and frozen down at -20°C. During sample collection rhesus macaques were anesthetized as mentioned above and bleed via the femoral vein at various time points. Monkey Peripheral blood mononuclear cells (PBMC) were isolated from anti-coagulated EDTA collected blood by density gradient centrifugation over histopaque (Sigma, St.Louis, MO, USA). White buffer layer was collected and treated with ammonium-chloride-potassium (ACK) lysis buffer to remove any red blood cells. Cells were then washed, counted, frozen and stored in liquid nitrogen until needed. Whole blood collected in red cap tubes were spun down at 2,000 rpm for 25 minutes for serum collection. Sera was then pulled off and stored at -80°C until needed.

2.2.3 Enzyme- Linked Immunosorbent Assay (ELISA)

Costar 96 well plates were coated (Costar flat bottom high binding) with 100ul of Concavalin A (50ug/ul diluted in PBS) per well for 1 hr at room temperature. Plates were then washed 2X with

PBS and coated overnight at 4°C with 50ng/ well of desired coating antigen (purified protein). Plates were then washed 4X with PBS and blocked with 200ul of 5% non-fat dry milk-PBS for 2 hr at RT. Plates were then washed 4X with PBS, and sera was added. For endpoint titers sera was serially diluted 1:2 across the plate with a starting dilution of 1:100. For cross binding ELISA, sera were pipette into the wells of a coated plate at 1:100. Sera were left on the coated plates for 1 hr at RT. Serum samples were then removed and plates were washed 4X with PBS. Then secondary antibodies (1:5000 dilution) were used to detect both mouse and non-human primate sera for 1 hour at RT (goat-anti-mouse IgG HRP/ goat-anti-Rhesus IgG HRP from Southern Biotech). Plates were then washed and developed using 3,3',5,5'-Tetramethyl-benzidine (Sigma) substrate to develop for half an hour. Reaction was then stopped using 50ul 1N Sulfuric Acid. The colorimetric change was measured at optical density of 450nm using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). End point titer for assay was determined as the reciprocal of the dilution at which the well's OD reading was above the mean plus two standard deviations of naïve sera.

2.2.4 *In Vitro* Neutralization Assay

Antisera were tested for the ability to neutralize virus infection in vitro using TZM-Bl cells indicator cells [200]. These cells express HIV receptors human CD4 (hCD4), human CCR5 (hCCR5), human CXCR4 (hCXCR4), and a luciferase reporter driven by the HIV-1 LTR. TZM-Bl cells were cultured in a 96 well plate in cDMEM with 10% fetal calf serum (10%) for 24 hours prior to infection with virus. Heat inactivated sera (56°C for 60 mins) was serially diluted (2 fold) and added to a standard quantity of virus (50TCID50). The sera/virus mixture was incubated at 37°C for 2 hr then added to the TZM-bl cells and incubated at 37°C for 48 h. Cell

lysates were harvested in lysis buffer (25mM Tris phosphate, pH=7.8, 2mM DTT, 2mM 1-2-diaminocyclohexane-N, N, N', N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Luciferase reagent was added was and the relative light units (RLU) were determined using a Femtomaster FB12 Luminometer (Zylux, Maryville, TN). The sera dilution necessary to neutralize virus was calculated by the following formula (RLU of virus only-RLU of cell only)/2+ RLU cell only.

2.2.5 Antibody Secreting Cell ELISPOT

Mice were sacrificed two weeks post final vaccination (week 6) and spleens were harvested and prepared into a single cell suspension. Briefly, PVDF membrane plates (Millipore, Billerica, MA,USA) were coated with purified consensus envelopes at (250ng/well) and kept at 4° C overnight. Plates were then washed with sterile PBS and blocked with cRPMI and placed at 37°C for ~3 hours. Media was then removed and the single cell suspension prepared was added to the plates at 10^5 cells per well. Plates were then incubated at 37°C for 48 hours. After incubation, plates were then washed with PBS and incubated for 2 hrs at room temperature with horse radish conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL USA). Plates were then washed as before and spots were developed at room temperature for 1 hour in the dark using detection substrate (NovaRED ^{TM},Vector Labs, Burlingame, CA, USA.). The plates were then washed extensively with DI water and allowed to dry overnight. Spots were counted using ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA.)

2.2.6 Viral Load Determination

Real time PCR-based SIV viral detection assay was used to determine the viral titers post-challenge as described in [201]. Briefly, 1 ml plasma was collected and concentrated via centrifugation (100,000g for 30 mins at 4°C) to pellet virus particles. Then 800µl of supernatant was removed and 200µl of pelleted virus solution was then added to NucliSen lysis tubes (bioMerieux, Hazelwood, Missouri, USA). Before RNA extraction, 10µl of $13X10^5$ particles of CM240 virus (as an external normalize) were added to the NucliSen tubes. RNA extraction was then performed as directed by manufacturer (bioMerieux, Hazelwood, Missour, USA). A standard curve was generatedfor each viral load assay using SIVmac251; RNA from the SIVmac251 dilution series was extracted along with the external normalizer. The primers and probes to 67-bp region of SIV gag DNA was used for amplification were forward primer SIVp15f1, reverse primer SIVp15r1, and probe SIVp15P 5′-joe AGCCTTTATAATACTGTCTGCGTCATCTGG-quencher 3′. For the external normalize CM240 forward primer envE2f (6928–6949) 5'-GGACAGGGCCATGTAAAAATGT-3', reverse primer 5'ENVe2r (7027–7002) 5'-TCTTCTGCTAGACTGCCATTTAACAG-3' and probe envEP (6965–6977) 5'-fam CACACATGGAATTAAGCCAGTGRTATCMACTCA-quencher 3'. 10µl of RNA was used in a 2-step TaqMan Gold RT-PCR reaction (Applied Bioscience, Carlsbad, California, USA). cDNA (10µl) generated by the RT-RCR reaction was then used for PCR using the ABI 7000 Gene detection system (Applied Bioscience, Carlsbad, California, USA)..

2.2.7 Anti- CD8 depletion by antibody administration

All animals in the Consensus M group were depleted of CD8+ T cells. The antibody M-T807R1 (NIH NHP Reagent Source, Beth Israel Deacones Medical Center, Boston, MA, USA) was administered subcutaneously (50mg/Kg) on day 0 (Day 70 post infection). CD8+T cell depletion was verified using TruCOUNT tubes (BD Bioscience, San Jose, CA, USA).

2.2.8 Absolute cell count using TruCOUNT tubes

Absolute T cell counts were done using 50 µl EDTA –collected whole blood placed into TruCOUNT tubes (BD Bioscience, San Jose, CA, USA). Cells were stained with a mixture of monoclonal antibodies CD3-PE (BD Bioscience), CD4-Amcyan (NIH NHP Reagent Source, Beth Israel Deacones Medical Center, Boston, MA, USA), CD8-APC-Alexa 750(eBio), CD45-PerCP (BD Bioscience, San Jose, CA, USA). Cells were incubated with monoclonal antibodies for 15 mins at RT followed by the addition of 450µl BD FACS lysing solution for 15 mins. Samples were then placed at 4°C and analyzed within 4 hr. Samples were collected in a BD LSR II flow cytometer and the data were analyzed by FlowJo software (Tree Start Inc. Ashland, OR, USA). T cell calculations were based on manufacture's equation [# events in cell population/# events in absolute count bead region x #beads/test (provided on package) /test volume].

2.2.9 ELISPOT

The number of anti-envelope (SF162p3 and consensus M) specific interferon gamma IFN-γ secreting cells were determined using the non-human primate enzyme-linked immunospot (ELISPOT) assay (R&D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-IFN-γ plates were incubated (30 min at 37°C) with (200µl) of sterile cRPMI. Plates were then seeded with peripheral mononuclear cells (PBMC $1x10^5$ cells) isolated at 14 post last vaccination or days 7 or 14 post infection. Cells were then stimulated for 48 hours with 30ug of Gag peptide pools representing SIV_{mac239} or Env pools representing either Consensus M or SF162p3 (overlapping peptides, 15-mers with11 amino acid overlap NIH AIDS Research and Reference Reagent program). As negative and positive controls cells were stimulated with Ova peptide and PMA/ionomycin (50ng/500ng) respectively. Plates were then washed 4X with R&D wash buffer and incubated at 4°C overnight with biotinylated anti-IFN-γ. Plates were washed 4X and incubated with streptavidin conjugated to alkaline phosphatase at 25°C for 2 hr. This step was followed by 4x washes with wash buffer and a1 hr incubation at 25°C with stable BCIP/NBT chromagen. The chromagen solution was discarded and plates were rinsed plate with deionized water and left to dry before being read. Plates were read using ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA.

2.2.10 Phylogenetic Tree

To create the phylogenetic tree the sequences of all envelopes used were submitted to the website phylogeny.lirmm.fr/phylo_cgi/index.cgi (managed by GForge project, funded by Reseau

National des Genopoles) as a fasta file. The sequences of the primary isolates were accessed from the genbank using their accession numbers. Using these sequences a tree was generated.

2.2.11 Statistical Analysis

Statistical tests were performed using Graph Prism software. Statistical significance of antibody test was determined by two-way analysis of variance (ANOVA) followed by the Bonferroni's post-test. Post-test was used to analyze differences between the vaccine groups. Significance was determined to be a $p<0.05$.

3.0 SPECIFIC AIMS

3.1 OVERALL OBJECTIVE AND RATIONALE

A major challenge in the development of an HIV/AIDS vaccine is the ability to overcome the virus' incredible envelope diversity. In these studies, two strategies were evaluated to overcome viral sequence diversity in Env. Polyvalent and consensus vaccine strategies increase the breadth of vaccine responses. Polyvalent vaccines consist of multiple immunogens to increase the epitopes available to stimulate a broadly–reactive immune response. The consensus vaccine is designed to capture the most common epitopes in an effort to decrease sequence variability between the vaccine and the wild type isolate. Therefore, immune responses elicited by a consensus immunogen are proposed to recognize multiple isolates resulting in a broadly-reactive adaptive response. Two separate group M vaccines are used in this study. The consensus M Env used in this study covers all the clades from HIV-1 group M, and the polyvalent vaccine covers clades A, B, C and E. The use of these consensus Env sequences was designed to address the problem of diversity within HIV strains by eliciting cross-protective immune responses. This is the first study to compare a single consensus envelope to a mixture of consensus envelopes. Comparing a consensus group M Env to a polyvalent clade consensus Env vaccine will address the question of whether a group M vaccine is sufficient to protect against a heterologous challenge. The characterization of the immune responses elicited by the vaccine and the

evaluation of the protection given by these immune responses will allow for insight into possible correlates of protection for HIV. Determining if a single consensus group M Env vaccine is sufficient for protection and eliciting an immune response that correlates with protection to viral challenge will impact the development of prophylaxis and therapeutic treatment in the HIV field. Having a single component vaccine will reduce cost of production and distribution of the vaccine. Therefore, the vaccine will be more affordable and accessible especially in developing regions such as Africa where the need for an effective vaccine is needed.

3.2 OVERALL HYPOTHESIS AND AIMS

Overall Hypothesis: A multi-clade vaccine expressing HIV-1 envelope proteins (con M or mixture of consensus envelope proteins) in their native structure will elicit a broad cross-reactive immune response capable of protecting from heterologous virus challenge.

Specific Aim I: To construct and characterize the Consensus Envelopes gp140 trimers

Hypothesis: All consensus gp140 envelopes will produce trimers and bind human soluble CD4

Specific Aim II: To determine the vaccine regimen of Consensus Envelopes that will elicit the highest Envelope titer to homologous vaccine

Hypothesis: The heterologous DNA prime containing envelope followed by purified gp140 envelope trimer will result in the highest homologous titer compared to DNA only or protein only vaccinations.

Specific Aim III: To evaluate the immunogenicity and efficacy of the Consensus M and Poly Consensus Vaccines in Non-Human Primates.

Hypothesis: Both vaccines will be immunogenic in NHP; however the Poly Consensus Vaccines have a better antibody breadth and be more efficacious.

4.0 SPECIFIC AIM I: TO CONSTRUCT AND CHARACTERIZE THE CONSENSUS ENVELOPES $_{GP140}$ TRIMERS

4.1 FOREWORD

A central question regarding development of a synthetic immunogen is whether its construction and design affects function or antigenicity of the native protein. This concern is especially critical in the case of consensus sequences, since they do not exist in nature. The quantity and quality of the antibody elicited by a vaccine depends on the antigen and how it is delivered to the immune system. Before antigen delivery can be investigated, antigen design needs to be addressed. The goal of this aim was to construct these envelopes and ensure they had similar functional and antigenic epitopes as wild type envelopes.

4.2 ABSTRACT

HIV envelope consists of two non-covalently linked subunits: gp120 (external subunit) and gp41 (transmembrane unit). The functional subunit of the HIV envelope is a trimer. Due to its critical role in viral entry, a tremendous amount of work is being done to develop a soluble envelope trimer as a vaccine candidate capable of eliciting anti-Env antibodies. In an effort to develop a preventative HIV envelope vaccine capable of overcoming the virus' high diversity, consensus

envelopes were designed. These consensus envelopes are based on the HIV clades A, B, C, E and group M. Viruses from group M, which encompasses clades A, B, C, E, have been shown to be responsible for most global infections. The sequences of the consensus envelopes have been truncated and modified to produce soluble envelope trimers. Consensus envelopes expressed as soluble trimers, retain their ability to bind HIV Env receptor CD4 and retained a well characterized cross-reactive epitope that is recognized by monoclonal antibody b12.

4.3 INTRODUCTION

In an infected individual, various isolates of HIV-1 exist as a mixture of closely related, but genetically distinct variants or *quasispecies* [202]. A combination of high rates of mutation, due to a highly error-prone replication, plus a viral production of millions of virus particles in a day illustrates the enormity of variants within each individual [203, 204]. In an effort to deal with the issues of viral diversity, strategies are being employed to construct centralized immunogen sequences that minimize the genetic distance to circulating viruses [205, 206]. Immunogens that are genetically closer to circulating variants are expected to elicit cross-reactive immune responses and lead to protection. Centralized sequences are influenced by sampling, computational inference, and recombination [207].

Sampling bias is a fundamental aspect that needs to be considered in the construction of centralized sequences. Ideally, the sample sequences should be representative of the phylogenetic distribution of the circulating strains and of their relative frequencies. Consensus (CON) and center-of-tree (COT) centralized sequences are heavily dependent on the sampling process, unlike the ancestral sequence (ANC) [208]. If a sample contains more sequences from

one clade, the resulting CON sequence would be more representative of that clade. To overcome sampling bias based on sequence availability, a layered consensus strategy was performed to design an H5 influenza HA CON (COBRA) vaccine by our group in the field of influenza [209]. The size and composition of the ideal target population will depend upon this co-diversification. The representative population used for generating the centralized gene could be based on a diverse set of parameters *e.g.* regional location of viral isolation, year of isolation, HIV clade or specific co-receptor usage. In general, the average distance between the sample sequences and the CON/COT sequence would likely be lower than the ANC sequence. A few studies have focused on the implications of different computational strategies that define the sequences of centralized genes. Kesturu *et al.* characterized the ability of three centralized representative sequences (CON, COT and ANC) to minimize the genetic distances for HIV-1 *env* sampled from 13 consecutive time points over an 8 year period in an infected individual [210]. During the first 5 years of infection, all three centralized sequences effectively minimized the genetic distance to the existing sequences. However when the centralized sequences were compared to later virus variants the ANC sequence was not as effective in minimizing genetic distance. While centralized sequences reduce genetic distance to circulating strains they may have an effect on the predicted 3-D structural properties, the number of antigenic epitopes, the affinity of MHC binding sites and number of N-glycosylation sites [211]. Recombination is an integral part of the HIV-1 life cycle with an estimated number of three recombination events occurring per genome replication [212]. Discovery that most infected cells harbor two or more different proviruses [213] give credence for recombination playing a central role in generation of HIV diversity [214, 215]. Due to the need for phylogenetic trees to determine COT and ANC recombination may mislead phylogenetic estimation of these two sequences [216, 217] . In contrast the CON

sequence has the advantage of not being biased by recombination since it is not dependent on phylogenetic trees.

The purpose of centralized genes in the use of more effective vaccines is based on the assumption that immunogenic epitopes of the virus will be constant to some extent. In addition, the evolutionary arms race between the virus population and the host immune system does not preclude the conservation of some key epitopes. In this study, both structural and genetic approaches were used to develop an envelope vaccine. Envelopes based on viruses responsible for most of the HIV global burden were designed using the consensus strategy of aligning full length amino acid sequences and selecting the most common amino acid in each position. Non-B HIV clades account for 88% of infections in high prevalence areas with clades C being dominant but including recombinants AE, AG [218], hence clades A,B,C and E and group M being chosen for Env consensus design. Individual consensus envelopes of clades A, B, C, E and a consensus envelope of group M, which includes the clades A, B, C, E, were engineered. For each consensus sequence, at least 100 envelope sequences representing viruses within that clade or group were aligned and the most common amino acid at each position was chosen. The envelope sequences were then truncated and modified via mutation of the cleavage site and addition of a bacteriophage trimerization domain to form envelope gp140 trimers upon expression in mammalian cells. Studies from our lab and others have produced wild type envelope trimers in the same manner [1, 2]. Upon purification, the consensus envelopes exhibited characteristics of wild type envelopes by producing stable trimers, bound to CD4 and contained a known cross-reactive epitope recognized by monoclonal antibody b12.

4.4 RESULTS

4.4.1 Design and Construction of Consensus Envelopes

The goal of this aim was to construct and characterize consensus envelope trimers. To accomplish this goal, the consensus envelope sequences were generated by selecting the most common amino acid at each position of a full-length envelope alignment. The consensus M envelope design using two hundred isolates sequences representing clades A, B, C, D, E, F and H. For the individual clade A, B, C and E consensus envelopes at least one hundred full length envelope sequences were used for each clade. The consensus envelope gene sequences were then truncated at the lysine (K) amino acid (Consensus M amino acid 665) to generate a gp140 protein that results in the expression of soluble envelope trimers. The Env cleavage site between gp120/gp41 was mutated by replacing the two arginines (R) at amnio acid sequence VVQSEKSA with serines (S) (Consensus M amino acids 490 and 493). To stabilize the truncated envelope trimers, the bacteriophage fibronectin domain (FT) was added to the end of the Env gp140 sequence (Figure 9), as previously described [1, 2].

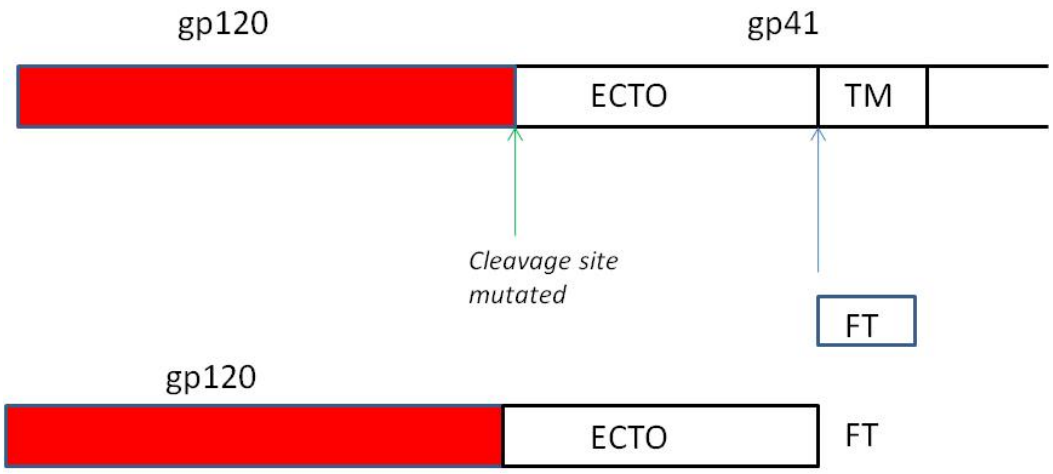


Figure 9. Diagram illustrating envelope modifications.

These modifications were based on previous studies by Bower et al ..[1] and Yang e. al[2]

4.4.2 Characterization of Consensus Envelopes

Supernatants from 293T cells were harvested following transfection with DNA plasmids expressing each of the consensus Env_{gp140} proteins. Individual consensus envelopes were then purified over lectin columns. Purified envelope trimers were separated by Invitrogen's NativePAGE Bis-Tris native gels (Figure 10). All consensus envelopes were detected by silver stain on native gel at ~480KD size indicating oligomerization as trimer proteins. Env dimers were observed primarily for consensus C, E and M Env_{gp140} proteins. To investigate the antigenic structure, the broadly reactive monoclonal antibody b12 was used to determine binding kinetics to each consensus envelope by BIACORE (Figure 11). The rate of association between the consensus envelope trimers to b12 was similar to the rate of association between b12 to primary envelope trimers of YU2, ADA and R2. The rate of dissociation of b12 from all the envelope trimers was similar, except for consensus B, which had a slower rate of dissociation. Each Env_{gp140} bound to the primary HIV receptor, human CD4 (hCD4) (Figure 12). Following immunopreciptation of soluble human CD4 both the pellet fraction (Env_{gp140}-hCD4 complex) and supernatant fraction (unbound proteins left in solution); were separated via SDS-PAGE. Western blots using antibodies to CD4 and the HIV envelope were used and both CD4 and consensus envelopes were detected in the pellet fraction. The supernatant and pellet fractions were stained by commassie blue for total protein. BSA was only observed in supernatant fraction and not pellet fraction (data not shown).

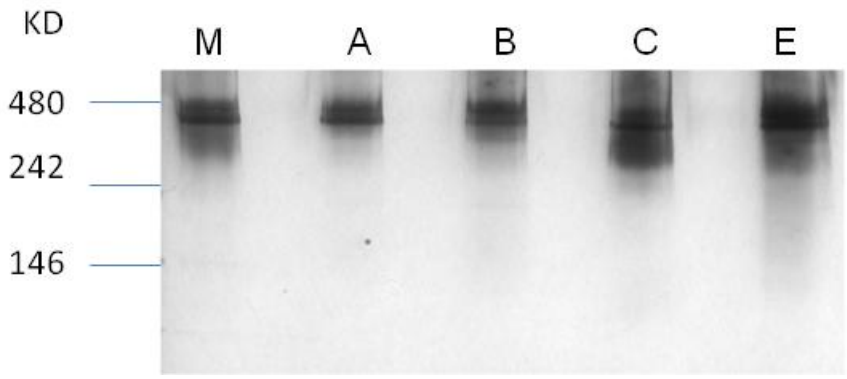


Figure 10 Native gel of lectin purified envelope timers

1μg of each purified consensus envelope was loaded onto a native protein gel and separated into their oligomer species by electrophoresis. All consensus envelopes were detected by sliver stain at ~480 kDa size. The top of the gel is labeled with the consensus envelope present in the lane and the protein ladder values are present on the y-axis.

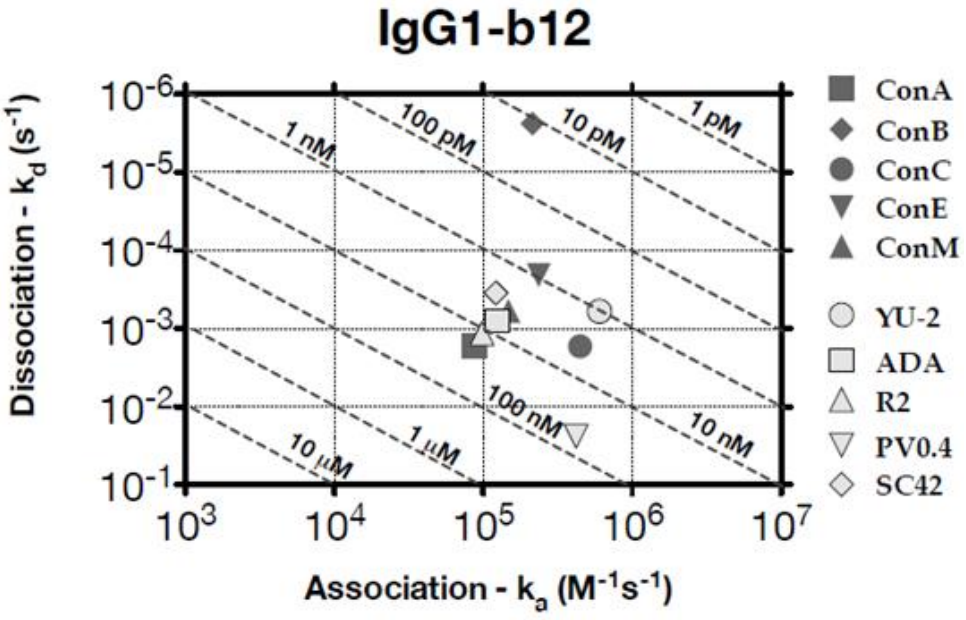


Figure 11.Graph showing interaction of envelope trimers with b12 monoclonal antibody.

Using surface plasmon resonance, the interaction of the consensus envelopes and b12 was investigated in solution. The x-axis gives the rates of association between b12 and the envelope and the y-axis gives the dissociation between the envelopes and b12. The consensus trimers are indicated by the darker symbols on the graph and the primary envelope trimers are indicated by the lighter symbols.

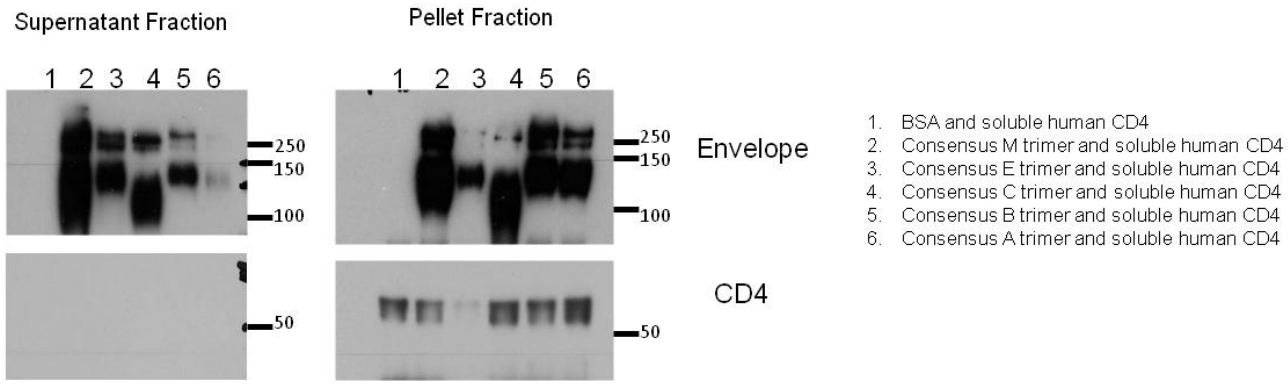


Figure 12 Western blot of supernatant and pellet fractions of CD4 binding Assay

The consensus envelopes and negative control BSA was pre-incubated with Histag soluble human CD4 at 37°C then mixed in with magnetic beads that were pre-incubated with the anti-his antibody. Using immunoprecipitation (IP), sCD4 and anything bound to it was pulled down in the pellet fraction. Supernatants and pellet fraction was analyzed by SDS-PAGE and western blot using anti-his and anti-envelope antibodies. Left panel: supernatants fraction. Right panel: pellet fraction (sCD4 and proteins bound). The protein ladder values on y-axis. The upper blots were probed with rabbit polyclonal anti-envelope antibody and the bottom blots were probed with mouse anti-human CD4 antibody (Clone RFT-4g mouse IgG, SouthernBiotech). Lane 1: BSA, Lane 2: Consensus M Env_{gp140}, Lane 3: Consensus E Env_{gp140}, Lane 4: Consensus C Env_{gp140}, Lane 5: Consensus B Env_{gp140}, Lane 6: Consensus A Env_{gp140}.

4.5 DISCUSSION

Consensus vaccines are a potential strategy to elicit broadly reactive immune responses. Consensus envelopes have been described previously and reported to be functional as measured by retention of cross-reactive antigenic epitopes [128, 130, 219]. However, the synthetic nature of consensus envelopes requires confirmation of functional and antigenic characteristics for each individual immunogen. Consensus sequences can influence 3-D structure of proteins but also the presence of antigenic epitopes, and number of N-linked glycosylation sites. In this aim we characterized the basic structural and antigenic properties of five synthetic HIV-1 gene products. Three questions were addressed: (i) do the modified consensus gene sequences generate trimeric Envelope proteins (ii) are these Envelope trimers functional and (iii) did consensus envelope retain antigenic epitopes?

Each of the consensus Env A, B, C, and E amino acid sequences were compared to consensus M. The consensus envelopes constant regions of gp120 had ~82-~93 % amino acid identity to the Con M. When the amino acid sequence identity for the consensus Env variable regions of gp120 were compared to Con M 80-89% with the distribution of the non-homologous amino acid spread across V1-V5. When the consensus M was compared to the publish group M Env ConS by Gao *et. al.* [219] the amino acid identity with the gp120 constant regions was ~98% and variable regions was ~93%. The amino acid sequence difference did not significantly affect the number N-linked glycosylation sites of consensus Env A, B, C, E compared to Con M; the number of glycosylation sites identified to be between 30-31 sites.

The data generated indicate that the five consensus envelopes express the same antigenic epitopes as Env from HIV-1 virus isolates. The envelopes contained linear epitopes present in wild type envelope as the polyclonal rabbit sera to HIV envelope IIIB was able to detect

envelopes via western blot. Compared to Env_{gp120} monomers, Env_{gp140} trimers may expose binding and neutralizing epitopes that are present only in envelope's quaternary state [220, 221]. The monomeric gp140 subunits formed trimers (Figure 10) that have similar antigenic properties as wild-type Env_{gp160s}, as demonstrated by attaching to human CD4. In addition, the monoclonal antibody b12 bound to each trimeric Env_{gp140}. The b12 antibody recognizes a conserved region on gp120 mapped to a discontinuous epitope overlapping the CD4 binding site [222]. Studies indicate that b12 monoclonal protect animals from viral challenge [223-225]. The results obtained with the consensus Env is this study was similar to that seen in previous consensus Env studies. Although antigenicity does not necessarily correlate to immunogenicity or efficacy, the presence of conserved epitopes indicates the possibility of the envelopes to induce a broadly reactive immune response.

5.0 SPECIFIC AIM II: TO DETERMINE THE VACCINE REGIMEN OF CONSENSUS ENVELOPES THAT WILL ELICIT THE HIGHEST ENVELOPE TITER TO HOMOLOGOUS VACCINE

5.1 FOREWORD

This chapter contains the results of experiments performed in Specific Aim II. The overall aim of this dissertation was to create an envelope vaccine capable of eliciting cross-binding antibodies to HIV-1 envelopes prior to viral challenge. Evaluating the immunogenicity of envelope trimers in a small animal model was essential before determining immunogenicity and efficacy in non-human primates. In addition, the vaccine regimen chosen has been shown to influence immune response elicited by vaccination.

5.2 ABSTRACT

Vaccine regimen has been shown to influence the outcome of HIV envelopes immunogenicity and efficacy. In this aim, four regimens were compared: DNA prime/boost, DNA prime/ Envelope trimer boost, Envelope trimer prime/boost or VLP prime/boost. To determine the most effective vaccine regimen to be used to evaluate all vaccines only the consensus M envelope was

used in the initial mice study. BALB/c mice were vaccinated at 0 and 3 weeks and sera were collected at week 5. Total IgG titer to the homologous antigen (consensus M) was used as the comparative measurement between the vaccines, and the regimen of Envelope trimer prime/boost was selected. In a follow up study mice were vaccinated via the Envelope trimer prime/boost regimen with either individual consensus antigens or a polyvalent mixture of consensus antigens (A, B, C, E). The antibodies elicited by vaccination did not differ in quantity or quality between envelope vaccines. Furthermore, the antibodies elicited reacted with not only the homologous vaccine antigen, but also binding breadth was extended to wild type envelope from multiple clades.

5.3 INTRODUCTION

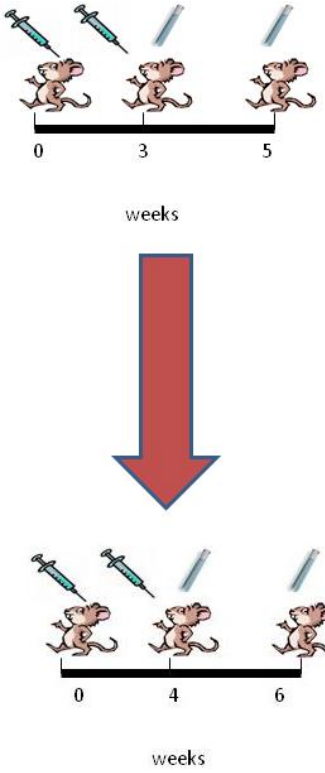
In developing a vaccine capable of eliciting a protective antibody response to Env, two aspects need to be considered: the antigen design and the immunization regimen used to deliver the antigen. Antigen design was covered previously (chapter 4); therefore, this chapter focuses on immunization regimen selection and antigen immunogenicity in mice. A number of strategies have been implemented in an effort to elicit an effective antibody response by vaccination. These strategies are (i) subunit protein [226, 227], (ii) viral vectors [228, 229], (iii) DNA vaccines [230, 231], (iv) viral vector plus protein [229], (v) DNA plus viral vector [97] and (vi) DNA plus protein [232]. After the STEP vaccine trial, concerns arose of potential vector enhancement of host susceptibility to HIV infection via immune activation [233]. Vaccine regimens with viral vectors were therefore not included as possible immunization regimens. The immunization regimens investigated were (i) DNA, (ii) DNA plus protein, and (iii) protein.

DNA only vaccination elicits specific antibody responses to HIV immunogens in animal studies and human clinical trials [230]. This success has been attributed to several positive aspects [234, 235]. First, the endogenous production and processing of the antigen allows for proper translation, native folding and normal post translational modifications. Secondly, the DNA is taken up by the cells at the site of vaccination making antigen production similar to live attenuated vaccines. Additionally, endogenous production of the antigen leads to efficient presentation to the immune system through class I and class II major histocompatibility complexes, thereby allowing for efficient T cell responses to the antigen. Lastly, unlike live-attenuated vaccines, DNA vaccines are non-replicative and non-integrative which improves the safety. However, DNA vaccines have been shown to have low *in vivo* transfection efficiency even after enhancement of DNA constructs such as codon optimization and manipulation of leader sequences and promoters [236, 237]. Despite the limitations *in vivo,* DNA vaccinations are effective at priming the immune system. Hence, the use of combination vaccines is an effective option to improve on DNA vaccination. One of the simplest approaches to modulations of DNA vaccination is following DNA priming with a protein boost [238]. Protein boost of a DNA prime has been reported to elicit higher antibody titers than DNA alone. In addition, better neutralizing antibodies have been reported after boosting a DNA prime with oligomerized protein antigens [239]. The initial immunization vaccine regimen used to elicit antibodies to HIV was protein vaccination, which consisted of subunit Env vaccines. These vaccines showed a moderate level of protection in monkeys but failed in human clinical trials [91]. With the advent of trimerized Env antigens, the use of a protein-only vaccination has reemerged from the initial HIV clinical trials. Over the last five years, protein-only vaccinations have elicited immune

responses that provided some level of protection against both homologous and heterologous SHIV mucosal challenges [240, 241].

Before the efficacy of any HIV vaccine can be investigated in the accepted model of monkeys, the immunogenicity is tested in small animals. Mice provide an *in vivo* model for which an antigen is characterized. Mice represent a less expensive animal model and the immunological tools are more readily available than in other rodents for investigating immune components. After vaccination in mice, the regimen that proved to be superior based on IgG titer was the protein vaccination. Using this immunization regimen, all consensus antigens elicited similar antibody responses either individually or as a polyvalent vaccine mixture (Poly consensus), which included consensus envelopes A, B, C and E. The only difference detected between the envelopes was the limited breadth of elicited anti-Env antibody respoenses by the consensus E Env: only three of the wild type envelopes were reactive. However, this limitation was not detected in the Poly consensus vaccine that will be used in the monkeys.

5.4 RESULTS



Mouse study 1: Determining Vaccine regimen using Consensus M vaccine

1. DNA/DNA
2. DNA/Protein
3. Protein/Protein (10ug each)
4. Protein/Protein (1ug each)
5. VLP/VLP
6. Alum
7. PBS

Mouse study 2: Determining Immunogenicity of consensus vaccines using the vaccine regimen protein (prime /boost) chosen based on study 1 results. 10µg of purified protein formulated with alum imject© was given to each mouse intramuscularly.

1. Consensus A
2. Consensus B
3. Consensus C
4. Consensus E
5. Consensus M
6. Polyvalent consensus (A,B,C,E)
7. PBS
8. Alum only

Figure 13: Schematic of the mouse studies performed in this aim.

The first mouse study was performed to determine the vaccine regimen that would elicit the highest total IgG to consensus M. The second study utilized the vaccine regimen chosen from study 1 (protein prime/boost) to evaluate the immunogenicity of all consensus vaccines and investigate whether combining the consensus envelopes reduce the immunogenicity of any individual envelope

5.4.1 Anti-Env responses between multiple vaccine regimens

Env-specific total IgG titer was measured two weeks after the final vaccination (Figure 14). After two vaccinations, there was one responder above the endpoint titer of 1:100 in both DNA prime vaccine groups. There were no responders in the VLP vaccine group or any of the control groups. In contrast, all mice in the two Env_{gp140} trimer only groups responded with end point titers greater than 1:1000. There was no significant difference between the endpoint titers of the mice vaccinated with 1μg or 10μg of envelope (p-value 0.0295). Serum of animals with detectable envelope responses was used to determine the IgG subtypes profile via ELISA (Figure 15). There was no difference in the pattern of IgG subtype elicited with either an Env_{gp140} trimer only regimen or a DNA only regimen. However, the DNA prime/Env_{gp140} trimer boost resulted in only an IgG_1 subtype being elicited.

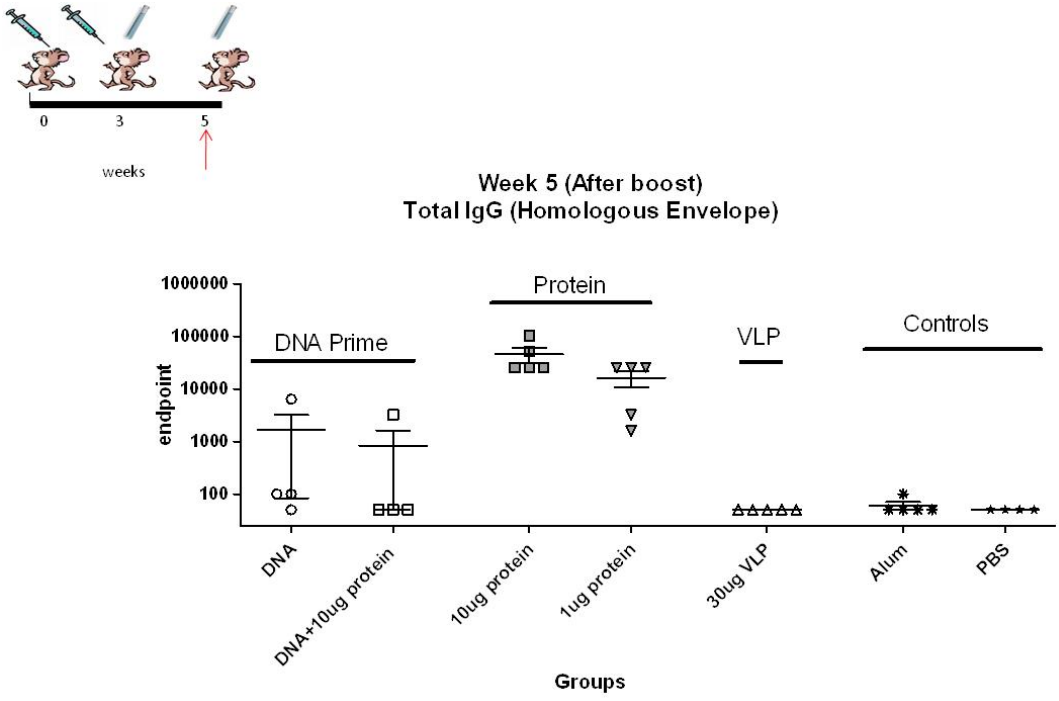


Figure 14 : Total IgG responses elicited to Consensus M

Mice were vaccinated with either a regimen of DNA only, protein only or a combination of DNA and protein. Animals were given 2 vaccinations intramuscularly, prime at week 0 and boost at week 3 and bled at week 5. Total IgG responses to homologous envelope were determined via ELISA to consensus M. The endpoint titer is described on the y–axis and the different vaccine regimens are described on the x-axis.

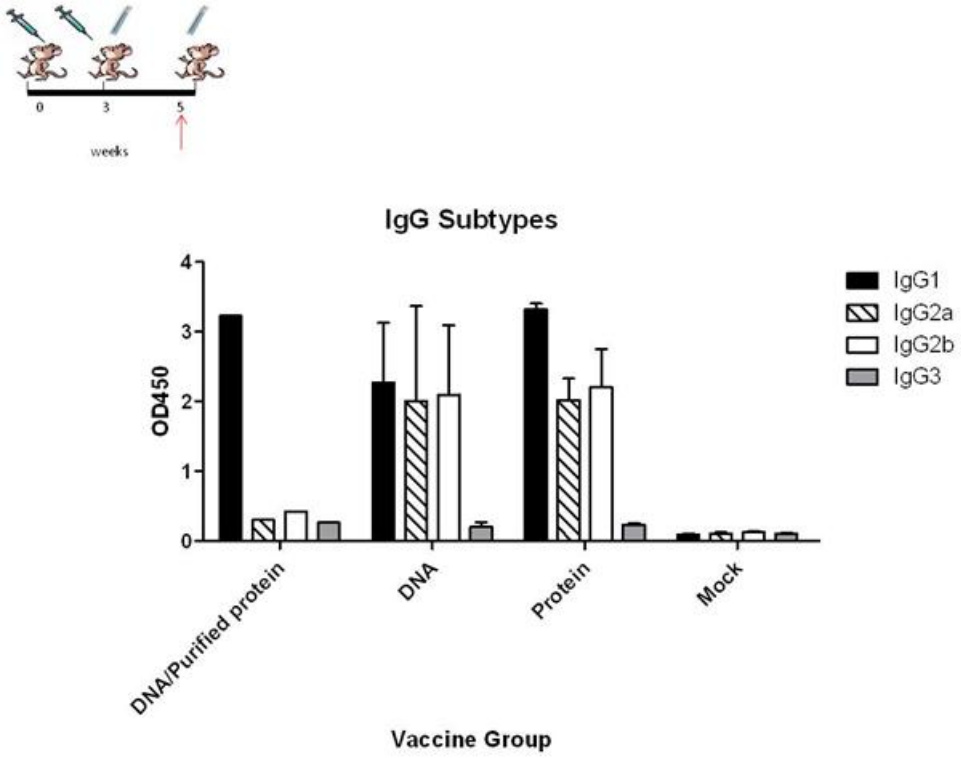


Figure 15 IgG subtypes elicited by various vaccine regimens

The sera of mice with detectable IgG envelope specific titers were used to determine the IgG subtypes elicited by vaccination included IgG 1, 2a and 2b. ELISA plates were coated with consensus M envelope and mouse serum was added at a dilution of 1:100. Secondary antibodies to mouse IgG subtypes conjugated to horse radish peroxidase were then used to detect the various IgG subtypes present in the mouse serum. The OD450 reading is given on the y–axis and the different vaccine regimens are described on the x-axis.

5.4.2 All consensus envelopes are equally Immunogenic

To verify the immunogenicity of all consensus envelopes prior to vaccinating non-human primates, purified consensus Env_{gp140} proteins were formulated with Imject® alum adjuvant and injected into BALB/c mice. Two weeks post the final vaccination, all mice vaccinated with primary consensus Env_{gp140} proteins representing clades A, B, C, and E elicited high titer antibodies that recognized all consensus envelopes (Figure 16A). Mice vaccinated with an equal mixture of primary consensus Env_{gp140} proteins (Poly Consensus) had similar anti-Env IgG titers against all four consensus Env antigens as mice vaccinated with Con M Env_{gp140} (Figure 16B). Mock vaccinated mice had no detectable anti-Env IgG responses. Investigation of antibody secreting cells showed similar numbers of cells secreting IgG after vaccination with either the Poly Consensus or Consensus M Env vaccines (Figure 17). All consensus Env vaccinations resulted in cross-binding antibody to a panel of wild-type Env proteins representing different clades of HIV-1 (Figure 18). There was no detectable difference in the number of wild type envelopes identified by any vaccine except by antibodies elicited by consensus E Env_{gp140}. Sera of mice vaccinated with consensus E Env_{gp140} recognized only three of the 11 wild type envelopes: 6535 (clade B), ZM214 (clade C) and 93TH975 (clade E).

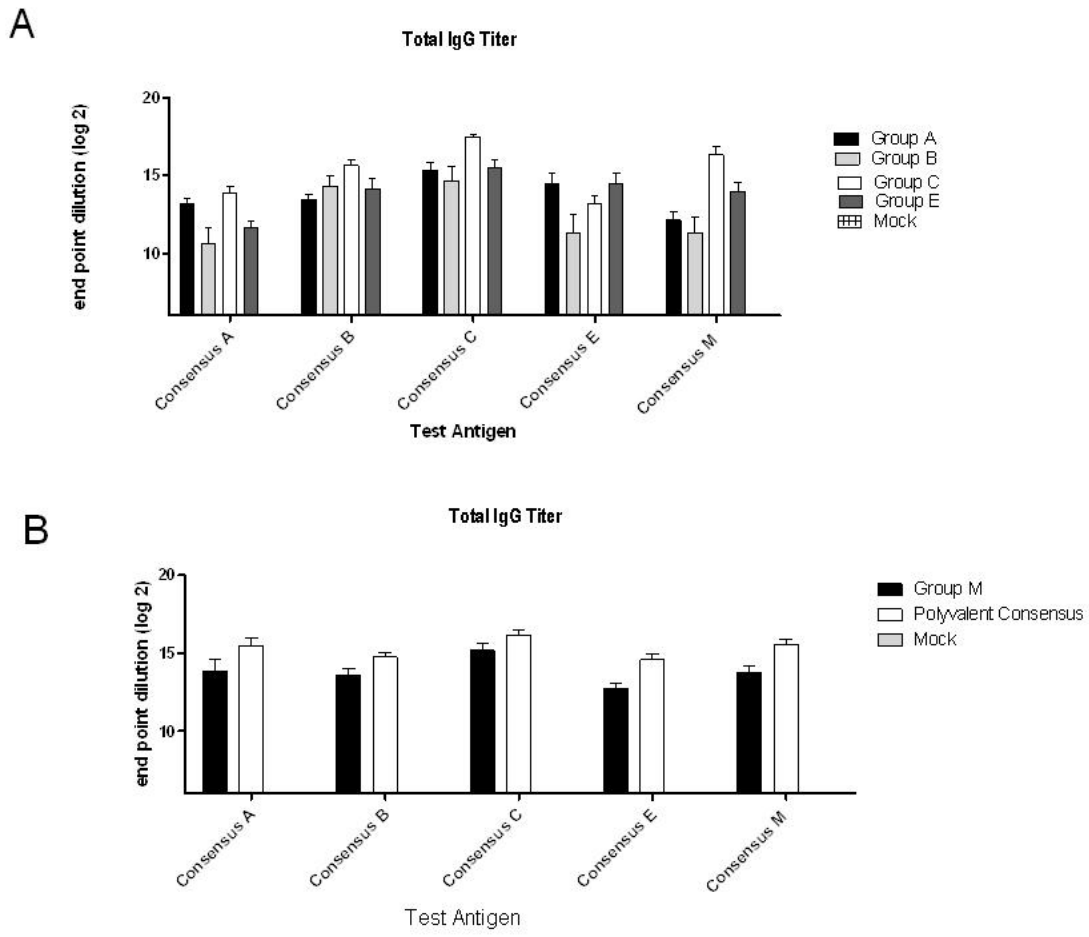


Figure 16: Mice seroconvert after vaccination with consensus vaccines

BALB/c mice were vaccinated at 0 and 4 weeks with blood collected at day 14 after final vaccination. Vaccines were formulated 10µg of purified protein with Imject® alum and delivered intramuscularly. Total IgG at week 6 was determined via ELISA for each vaccine group. Values represent the geometric mean titer (+95% confidence interval) of log2 transformed titers. A) Endpoint titers of animals vaccinated with individual clade consensus envelope trimers (A, B, C, E). B) Endpoint titers of animals vaccinated with group M consensus envelope trimer and polyvalent vaccine (equivalent amounts of clade consensus envelope trimers). The endpoint titer is described on the y–axis and the identified Env_{gp140} trimer antigen used as coating antigen is described on the x-axis.

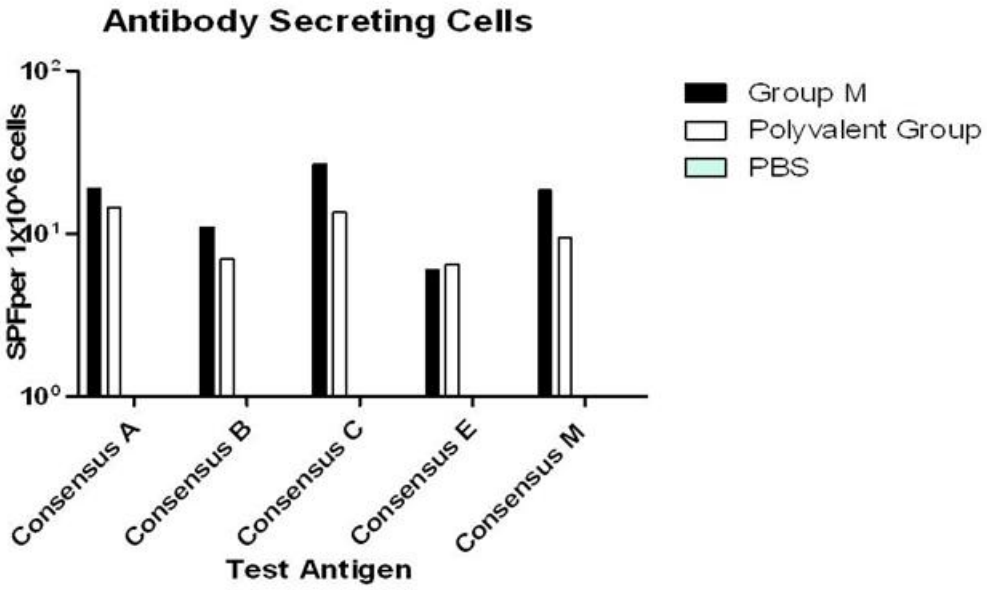
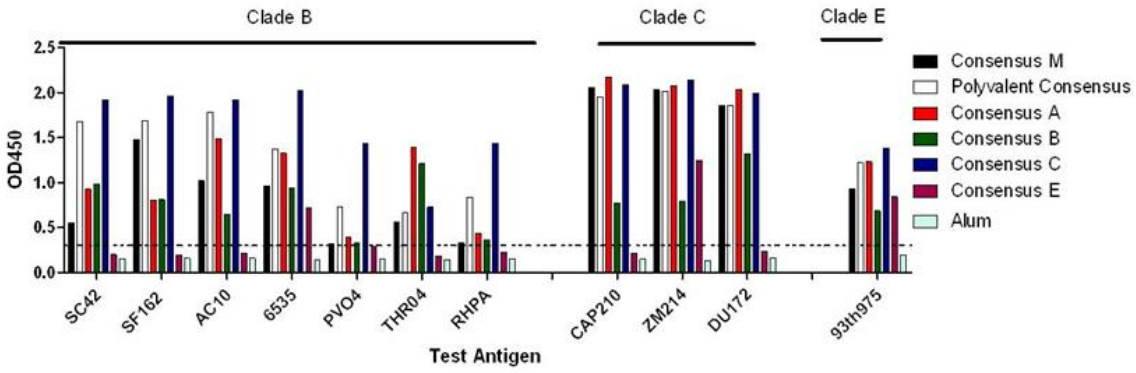


Figure 17: Similar number of Antibody Secreting (ASC) detected with consensus Env

Mice splenocytes were used to evaluate the presence of ASC seven days after final vaccination. Plates preincubated overnight with the different envelopes proteins (A, B, C, E, and M) were loaded with single cell suspension of splenocytes. After 48 h incubation at 37 °C with a mouse IgG conjugated with horse radish peroxidase (HRP) antibody, the complex was detected using ImmPact NovaRED peroxidase substrate (NovaRED ™,Vector Labs, Burlingame, CA, USA.) to develop Antibody Secreting Cells specific to the envelope coated on the plate. These spots were then counted by the ImmunoSpot ELISPOT reader. The number of cells detected per $1x10^6$ splenocytes on the y–axis and the identified Env_{gp140} trimer antigen used as coating antigen is described on the x-axis.



Vaccine	No. of envelopes from clade B	No. of envelopes from clade C	Clade E envelope
Consensus M	6 of 7	3 of 3	1 of 1
Polyvalent	7 of 7	3 of 3	1 of 1
Consensus A	7 of 7	3 of 3	1 of 1
Consensus B	5of 7	3 of 3	1 of 1
Consensus C	7 of 7	3 of 3	1 of 1
Consensus E	1of 7	1 of 3	1 of 1
Alum/mock	0 of 7	0 of 3	0 of 1

Figure 18. Consensus vaccines elicit cross-binding antibodies

BALB/c mice were vaccinated at 0 and 4 weeks with blood collected at day 14 after final vaccination. Vaccines were formulated 10µg of purified protein with Imject® alum and delivered intramuscularly. Serum IgG was detected by allowing anti-Env antibodies to bind to primary gp120 envelopes from either clades A, B, C and E. A positive titer was determined when the antisera detected the Env at a dilution that was 2X over the antisera collected from control, non-vaccinated animals. The OD450 reading is on the y–axis and the identified Env_{gp140} trimer antigen used as coating antigen is described on the x-axis.

5.5 DISCUSSION

The multiple strategies used in this chapter have been performed by our group and others with various antigens including envelope [1, 240, 242]. However, the antigen influences the elicited antibody and there was a necessity to directly compare multiple immunization regimens using the synthetic consensus Env_{gp140} vaccines. The results from these studies identified the protein vaccination as the vaccine regimen that induced the highest total IgG Env responses. The protein vaccine induced the highest total IgG titer to homologous envelope and induced the same IgG subtype profile as DNA only vaccination. The IgG subtypes elicited by vaccination included IgG 1, 2a and 2b. Mouse IgG 2a and 2b are potent inducers of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity [243]. Active and passive immunization studies have implicated both of these functions as possible mechanisms by which antibodies mediate protection [224, 244, 245]. The presence of these IgG subtypes further supported the protein immunization regimen for the consensus Env antigens. The poor responses seen in the immunization regimen of DNA prime plus protein boost was not expected. The DNA used is the same plasmid used to produce the purified protein, hence it is known to express. The poor responses seen with DNA vaccination could be a result of the dose of DNA used and or the mode of DNA vaccination.

The vaccines that are being considered for use in monkeys are the consensus M and the polyvalent consensus vaccines (A, B, C, and E). The monkey study was only powered to evaluate two vaccines and therefore the immunogenicity of individual consensus envelopes A, B, C, and E had to be evaluated in mice. In addition, to ensure that one envelope is not significantly more immunogenic causing a bias of the elicited immune response, each Env antigen was evaluated individually and in combination as the intended polyvalent mixture. In addition, the

consensus Env antigens elicited broadly binding antibodies that reacted to a panel of wild type Envs. The wild type envelopes were chosen because they were transmitted mucosally, and the isolates used the CCR5 coreceptor. These features are key characteristics of viruses involve in transmission. The only Env_{gp140} vaccine that elicited a narrow anti-Env binding breadth was consensus E Env_{gp140}, which detected only three of the eleven envelopes tested. However, the inclusion of consensus E Env_{gp140} protein in the polyvalent vaccine did not affect the breadth of the antibody responses elicited by vaccination. On the contrary, it supports the use of polyvalent mixtures as a strategy for broadening a vaccine's immune responses beyond a single immunogen. The fact that the consensus E Env had the least amino acid identity with consensus M (~83%) may explain its limited envelope breadth to other clades. Using consensus M is the immunogen with the least diversity to primary HIV isolate envelopes consensus E was the most divergent from primary HIV isolate envelopes, especially across clades. The results generated from vaccination in mice confirm the immunogenicity of the consensus Env_{gp140} trimer vaccines and allows for the evaluation of these vaccines for immunogenicity and protective efficacy in monkeys. The results also confirm the use of consensus antigens to broaden antibody responses as previously reported [128, 219].

6.0 SPECIFIC AIM III: TO EVALUATE THE IMMUNOGENICITY AND EFFICACY OF THE CONSENSUS M AND POLY CONSENSUS VACCINES IN NON-HUMAN PRIMATES

6.1 FOREWORD

With the absence of known correlates of protection for HIV, a vaccine's efficacy can only be determined by challenge. In this chapter two vaccines are being evaluated for immunogenicity and efficacy in monkeys. The vaccine was designed in an effort to produce a breadth of cross-clade binding envelope specific antibodies prior to challenge. Specific Aim III was design to address the question of vaccine efficacy and the results of this aim is covered in the following chapter. The accepted model to determine vaccine efficacy is the non-human primate, therefore this is the animal model being used..

6.2 ABSTARCT

The development of a preventative HIV/AIDS vaccine is challenging due to the diversity of viral genome sequences, especially in the viral envelope. Since it is not possible to directly match the vaccine strain to the vast number of circulating HIV-1 strains, it is necessary to develop an HIV-1 vaccine that can protect against a heterologous viral challenge. Previous studies from our group

demonstrated that a mixture of wild type clade B Envs were able to protect against a heterologous clade B challenge more effectively than a consensus clade B Env vaccine. In order to broaden the immune response to other clades of HIV, in this study rhesus macaques were vaccinated with a polyvalent mixture of purified HIV-1 trimerized consensus Env_{gp140} proteins representing clades A, B, C, and E. The elicited immune responses were compared to a single consensus envelope representing all isolates in group M (Con M). Both vaccines elicited anti-envelope IgG antibodies that bound an equal number of HIV-1 Envs representing clades A, B and C. In addition, both vaccines elicited antibodies that neutralized the $HIV\text{-}1_{SF162}$ isolate. However, the vaccinated monkeys were not protected against $SHIV_{SF162p4}$ challenge. These results indicate that consensus envelope vaccines, administered as purified Env trimmers, elicit antibodies that bind to Envs from strains representing multiple clades of HIV-1, but these vaccines did not protect against heterologous SHIV challenge.

6.3 INTRODUCTION

Human Immunodeficiency Virus (HIV)/Acquire Immunodeficiency Syndrome (AIDS) have been a global public health issue for ~30 years. The World Health Organization (WHO) in 2011 reported 2.7 million new HIV infections with 34 million people living with the virus worldwide [5]. Despite the effectiveness of highly active anti-retroviral therapy (HAART) [246], a long time goal to solving the AIDS crisis is an effective vaccine [247]. One of the greatest struggles for developing a preventative HIV vaccine is overcoming the diversity of viral isolates [248]. The envelope sequences can differ up to 35% between clades and ~15% within a specific clade [249]. Viruses classified as clade B are responsible for $\geq$40% of infections in the Americas and

Europe, but in Asia and sub-Saharan Africa, where most new infections are recorded each year, other clades are dominant. Most new infections in these regions are classified as clades A, C, or A/E viruses [250, 251]. Any HIV vaccine that will prevent infection must be able to overcome the diversity of HIV sequences.

Two strategies that have been used in vaccine design to overcome the HIV sequence diversity are polyvalent mixtures and consensus antigens [104, 252-254]. Polyvalent vaccines increase breadth by including several epitopes by adding multiple copies of a target(s) into a single formula. Polyvalent vaccine strategies have been employed to increase the breadth of the humoral and cellular immune responses [200, 255]. Previous publications with polyvalent mixtures of envelopes or HIV proteins (Gag-Pol, Tat and trimeric envelope) reported a degree of protection against heterologous challenge [200, 241]. Another strategy uses consensus antigens as immunogens. These vaccines rely on a centralized antigen designed to reduce sequence diversity by using the most common amino acid at each position of the protein. Consensus vaccines are designed to reduce the genetic differences between the vaccine and the primary isolate. Consensus antigens have been shown by our lab and others to increase the breadth of immune responses [135, 256-258]. Previously, our laboratory compared the ability of a consensus clade B (ConB) Env VLP vaccine and a polyvalent clade B (PolyB) Env VLP vaccine to protected rhesus macaques against a vaginal $SHIV_{SF162p4}$ challenge [200]. However, there were undetectable levels of anti-Env IgG or neutralizing titers in these vaccinated animals prior to challenge.

In this study to overcome the diversity in envelope sequences and to design a more effective AIDS vaccine, consensus envelope sequences were designed for 4 clades of HIV-1 (A, B, C, and E), as well as a single consensus Env representing isolates from all of Group M. For

the first time in the same study consensus Env sequences were used in a polyvalent vaccine mixture, and compared to a Con M Env, to assess the ability to elicit a broadly reactive anti-Env immune response. To elicited immune responses to Env prior to challenge, rhesus macaques were vaccinated with trimerized consensus Env_{gp140} immunogens in a either polyvalent mixture representing clades each of the 4 clades or as a single consensus M envelope. The immunological responses of the polyvalent mixture were compared to that of the single Con M Env_{gp140} vaccine. Even though both vaccines elicited a broader anti-Env immune responses against multiple clades of HIV, however, neither vaccine strategy efficiently protected monkeys against a $SHIV_{SF162p4}$ challenge.

6.4 RESULTS

6.4.1 Vaccination of Non-Human Primates with Consensus Envelopes

To determine the ability of the vaccines to induce a protective response in non-human primates, rhesus macaques were vaccinated with either Con M or the polyvalent consensus mixture. Monkeys were administered 3 vaccinations of Env_{gp140} formulated with Imject® alum adjuvant. Mock vaccinated monkeys were administered PBS formulated with adjuvant (Table 4) Monkeys vaccinated with Con M or polyvalent consensus had anti-Env antibody titers greater than 1:400 against all consensus envelopes following the three vaccinations (Fig. 19A). Collected sera were tested for the ability to bind to a set of primary envelopes representing clades A, B, C, and E (Table 5). These diverse R5-tropic envelopes represent viruses were isolated from individuals located in different areas of the world following various modes of transmission. Most of the

viruses were isolated within a few weeks of transmission. The diversity of the envelopes chosen, as well as their similarity to the consensus envelopes, is displayed in the phylogenetic tree where envelopes cluster into their identified clades (Fig. 19B). Also, the consensus envelopes clustered with the envelopes from their representative clades. The consensus M did not cluster with any one clade, but as expected, was located in a more central position on the tree.

Both Con M and polyvalent consensus Envgp140 vaccines elicited anti-Env antibodies that recognized envelopes from clade A, B and C. However, the envelopes SC42, THRO4, PVO4, (clade B), DU172 (clade C) and 93TH975 (clade E) were not significantly recognized by sera collected from vaccinated animals (Fig. 19C). Overall, there was no binding preference of the elicited anti-Env antibodies to primary envelopes based on clade, location, or year of envelope isolation.

Table 4: Vaccine Groups and Vaccine given

Each animal was vaccinated 3 times week 0, 4 and 8. Each vaccine was given in combination alum imject© as an adjuvant. The table gives the vaccine groups, the identification number of animals in each group and the vaccine and treatment (in the case of group 3) received by the animals

Vaccine Group	Animal Numbers	Vaccine Given	Other Treatment
Group 1	N1,N2,N3,N4	Adjuvant only	
Group 2	P1,P2,P3,P4	Mixture of consensus A,B,C,E Env $_{gp140}$	
Group 3	M1,M2,M3,M4	Consensus M Env $_{gp140}$	Depletion of $CD8^+$ T cells

Table 5: Information of envelopes used for assays:

Primary envelopes from various clade was chosen based to have a diversity of locations and mode of mucosal transmission. Envelopes were used to test the presence of binding and neutralizing antibodies elicited by vaccination of consensus vaccines in non-human primates. SF162 was included because it is the challenge virus envelope

Env ID	Clade	Location	Mode of Transmission	Length of Infection	Mo/yr isolated	Coreceptor
Du172.17	C	South Africa	M-F	12 weeks	Nov-98	R5
ZM214M.PL15	C	Zambia	F-M	<13 weeks	Jul-03	R5
CAP210.2.00.E8	C	South Africa	M-F	5 weeks	May-05	R5
CHN19	C	China				R5
HIV16936-2__EF117270	C	India	F-M	1 week	Nov-00	R5
Q168.ENV.B23	A	Kenya	M-F	1week		R5
Q461.ENV.C2	A	Kenya	M-F	4 weeks		R5
HIV env 6535 clone 3	B	USA	M-M	6 weeks	Mar-95	R5
PVO clone 4	B	Italy	M-M	4 weeks	Jan-96	R5
pRHPA 4259 clone7	B	USA	M-F	<8 weeks	Dec-00	R5
pTHRO4156 clone 18	B	USA	M-M	1 week	Aug-00	R5
SC 422661.8	B	Trinidad	F-M	4 weeks	Jan-95	R5
SF162	B	USA				R5
93TH975-15	E	Haiti	F-M	<18 weeks	Jan-93	R5

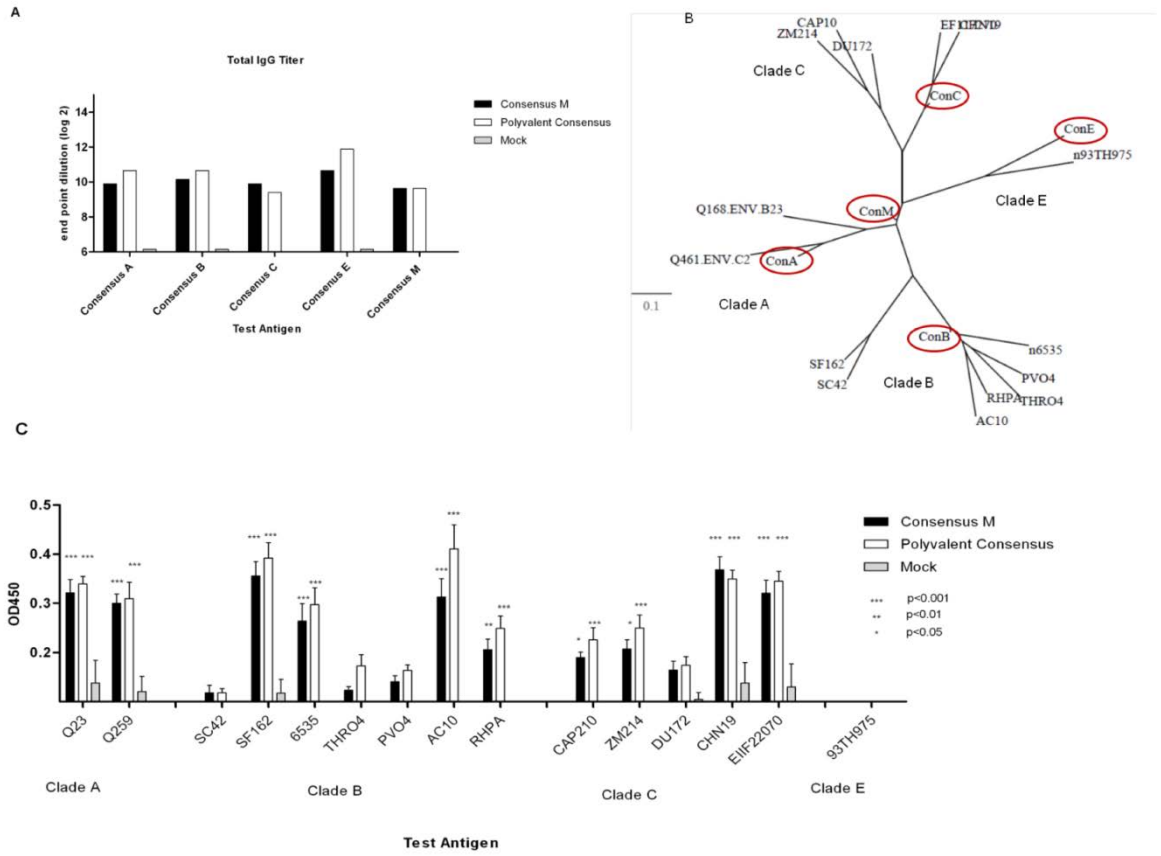


Figure 19: Binding breadth of vaccinated NHPs sera.

NHP were vaccinated at weeks 0, 4 and 8 with blood collected 14 days after each vaccination. Vaccines were formulated with 300μg of purified protein and Imject® alum and delivered intramuscularly. A) Sera collected on day 35 were used to determine total IgG via ELISA for each vaccine group. Bar values represent the geometric mean titer (+95% confidence interval) of log2 transformed titers. The endpoint titer is described on the y–axis and the identified Env_{gp140} trimer antigen is described on the x-axis. B) The unrooted phylogenetic tree was produced using Phylogeny.fr web service and the 14 HIV-1 envelope sequences based upon the list of envelopes in table 5 showing. The envelopes were from clades A, B, C, E from 1993-2005. The clades are indicated on the tree and the consensus envelopes are circled. C) At day 35 post-vaccination, anti-Env IgG was detected in the serum samples (1:100 dilution) against a panel of primary Env_{gp120s} from clades A, B, C and E via ELISA. Bar values represent the geometric mean titer (95% confidence interval) at an OD450. The OD450 values are displayed on the y-axis and the Env_{gp120s} used are listed on the x-axis. A two-way ANOVA with Bonferroni's post-test was used to evaluate Statistical significance between the vaccines for each test antigen. A *p*-value of less than 0.05 was considered significant. Significant seen is in relation to mock vaccinated animals.

6.4.2 Responses to Challenge Envelope SF162

At 2 weeks after final vaccination (day -14 prior to challenge), anti-Env_{SF162} IgG antibodies were detected in monkeys vaccinated with either vaccine (Fig. 19C). In addition, these antibodies were able to neutralize the ability of HIV_{SF162} to infect cells in vitro (Fig. 20A). All vaccinated monkeys had a neutralizing titer of 1:40, except for monkey M4 that had a titer of 1:320. Two weeks following challenge, only monkey P4 had an increase in neutralizing titers. There were detectable neutralization titers in 3 of the 4 mock vaccinated monkeys two weeks post-challenge (Fig. 20A). No monkey had antibodies that recognized a set of overlapping SF162 envelope peptides. These pools of peptides represented the SF162 Env regions V1/V2, V1 only, or V2 only (data not shown). Interestingly, there was no detectable INF-γ envelope or gag-specific T-cell responses prior to challenge or 7 days post-challenge. At day 14 post-challenge, monkeys vaccinated with the polyvalent consensus vaccine had higher number of T cell response compared to monkeys vaccinated with Con M vaccine (Fig. 20B). However, differences in number of T cell responses between the animal groups were not significant difference.

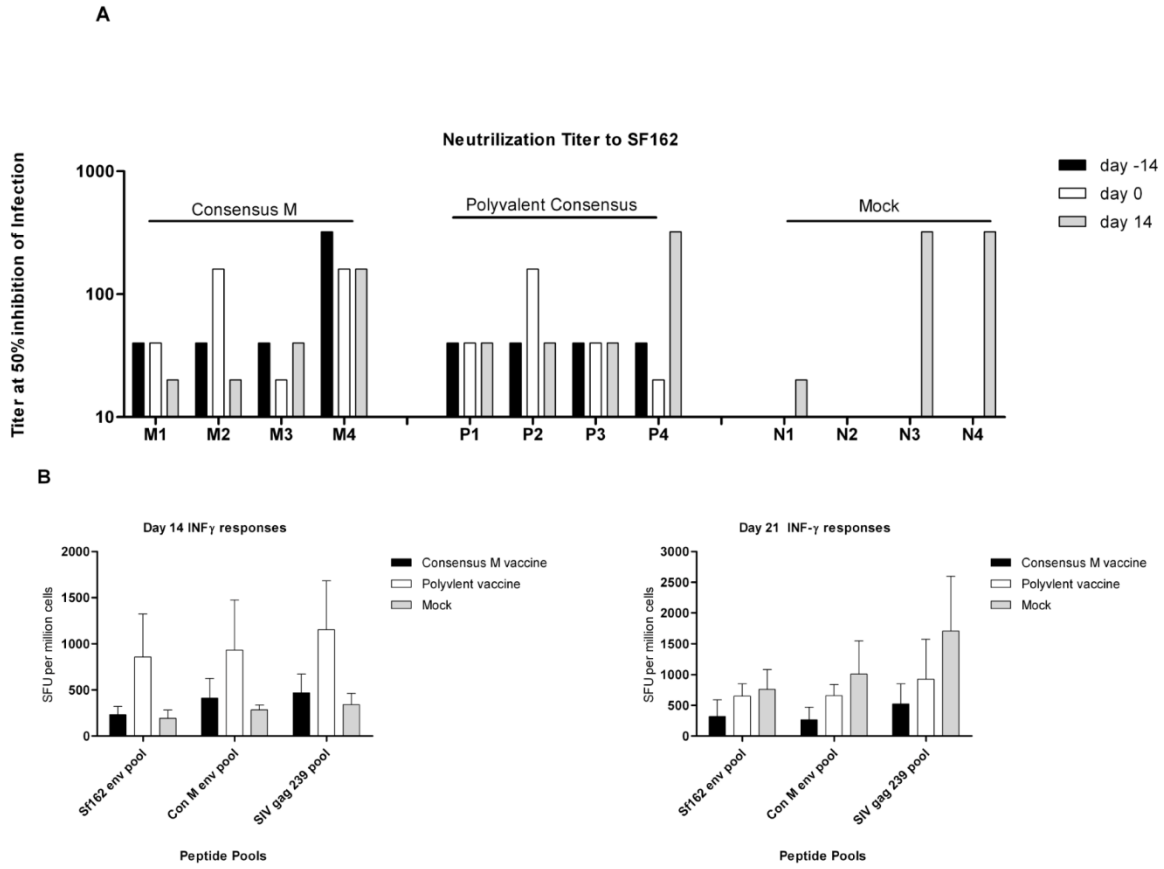


Figure 20 Neutralizing titers to the SF162 envelope and post challenge cellular responses.

A) Neutralization of HIV_{SF162} by serum collected at day 21 and 35 post-vaccination and 14 post-challenge. The sera dilution at which 50% of virus infection is inhibited is displayed on the y-axis. Neutralizing titers for individuals monkeys are listed on the x-axis by each vaccine group. B) INF-γ ELISPOTs were performed using monkey PBMCs collected 14 (left panel) and 21 (right panel) days post-challenge. $1x10^5$ PBMCs were stimulated with 30ug of Gag peptide pools representing SIV_{mac239} or Env pools representing either Consensus M or SF162p3 (overlapping peptides, 15-mers with11 amino acid overlap NIH AIDS Research and Reference Reagent program. Spot forming units (SFU) per one million PBMCs are listed on the y-axis and the peptide

6.4.3 $SHIV_{SF162}$ challenge

To evaluate the protective efficacy of each vaccine, monkeys were challenged rectally four weeks after final vaccination with $SHIV_{SF162p4}$ (640_{TCID50}). All mock vaccinated and polyvalent consensus vaccinated monkeys were infected following challenge (Fig. 21A and B). Viral loads peaked at day 14 post-challenge at ~1 X $10^{\wedge 6}$ RNA copies/ml and then declined to undetectable levels between days 40-80 post-infection. Two out of fours monkeys (M1 and M4) vaccinated with Con M vaccine had no detectable virus at any time point post-challenge (Fig. 21C). Monkeys M2 and M3 had a similar viral pattern as mock vaccinated monkeys with a peak at day 14, followed by a rapid decline.

To determine whether Con M vaccinated monkeys completely sterilized infection, at day 70 post-challenge all Con M vaccinated monkeys were depleted of CD8+ cells by administering monoclonal antibody M-T807R1 intravenously [245]. Seven days following antibody administration, no CD8+ cells were detected in the peripheral blood that was sustained for an additional 18 days (Fig. 22A). Previously infected monkeys M2 and M3 had a re-emergence of virus during this CD8+-depletion period (Fig. 22B). Monkey M4 who was initially averimic after challenge had an emergence of virus after CD8+-depletion. In contrast, monkey M1 maintained undetectable viral loads following CD8+ depletion.

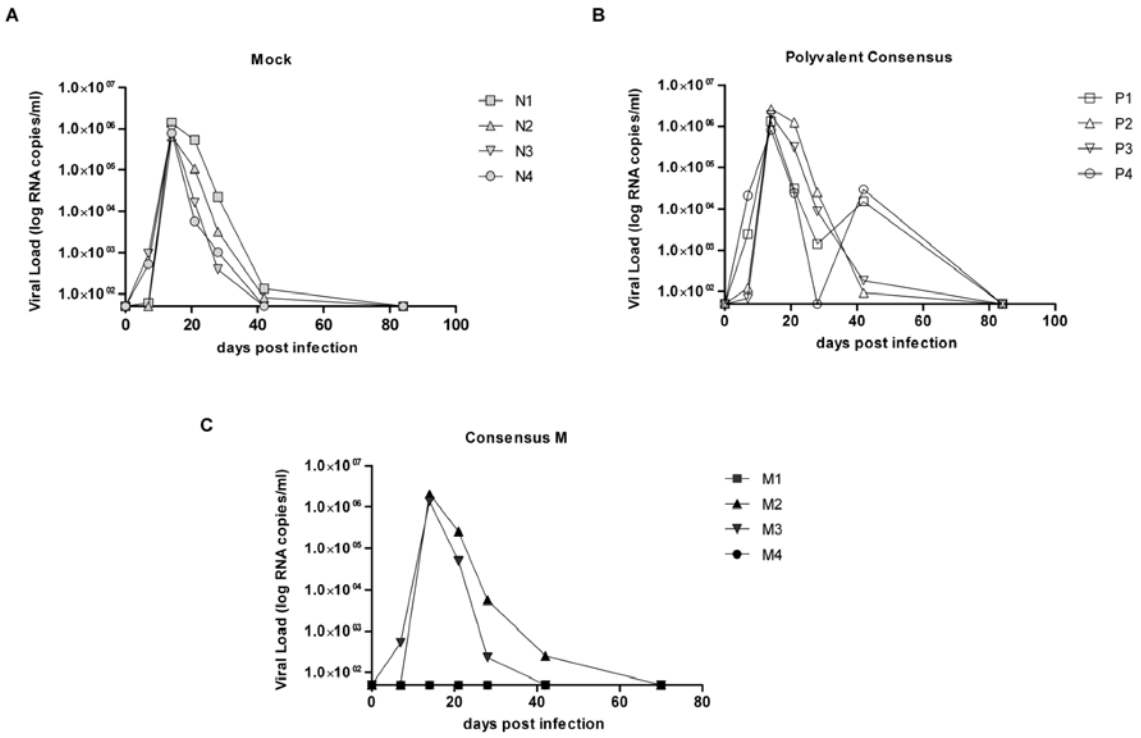
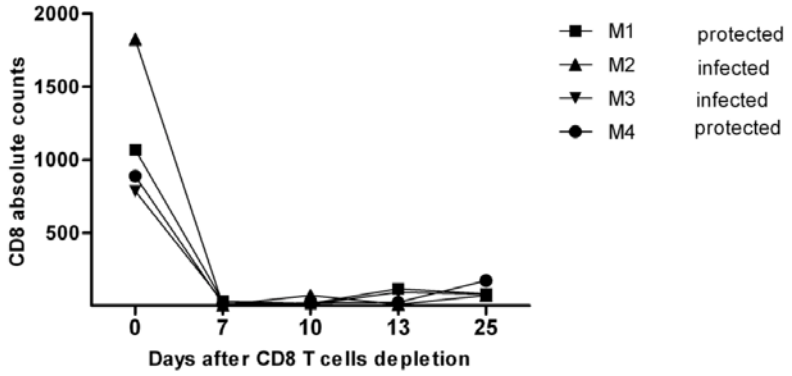


Figure 21 $SHIV_{SF162}$ virus challenge:

Monkeys were challenged rectally with 640 $TCID_{50}$ SHIV SF162p4 at week 12 post initial vaccination. Viral titers were determined from collected sera. Viral titers for each individual monkey are displayed as RNA copies/ml of blood on the y-axis by days post challenge on the x-axis. A) Mock vaccinated animals (Imject© adjuvant only). B) Polyvalent Consensus Env_{gp140} vaccine C) Consensus M Env_{gp140} vaccine.

A



B

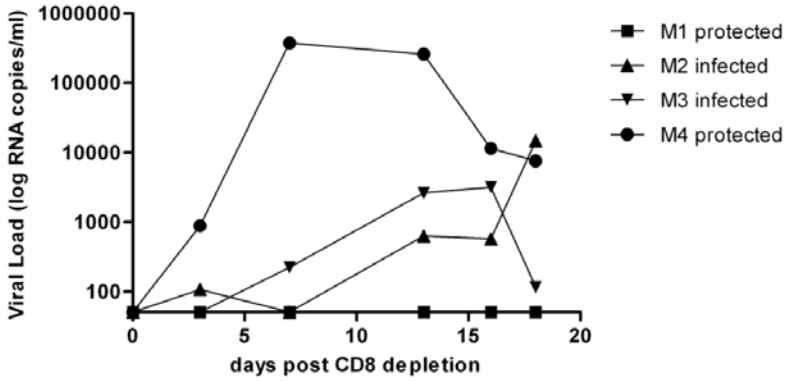


Figure 22 Confirming Vaccine efficacy

Animals vaccinated with consensus M Env_{gp140} were all depleted of CD8+ cells by administering the M-T807R1 antibody subcutaneously (50mg/Kg) at day 0 (day 70 post challenge). A) Number of CD8+ cells following antibody administration over the 25 day period of observation. B) Viral titers for each individual monkey are displayed as RNA copies/ml of blood on the y-axis by days post challenge on the x-axis.

6.5 DISCUSSION

HIV-1 envelope only vaccines have been used effectively to protect monkeys against a homologous SHIV challenges [259, 260]. Our hypothesis put forth in this study was to design an envelope based vaccine to elicit broadly reactive antibody responses to multiple envelopes representing different clades of HIV-1 and to protect against a virus with a heterologous envelope to the challenge virus. For real-world situations in humans, the ability to match the gene sequences used in the vaccine to the possible exposure virus is not possible. Therefore, studies that use a matched envelope in the vaccine to the challenge strain is appropriate for proof-of concept studies, but our group set a more challenging goal to protect against a challenge virus with a mismatched vaccine with a limited number of vaccinations. We report here that each consensus sequence representing clade A, B, C, E, in a polyvalent mixture or as a single consensus envelope representing the entire Group M elicited anti-Env antibodies that bound to a broad panel of HIV-1 envelopes. However, the elicited immunity did not prevent infection by $SHIV_{SF162p4}$.

We chose to use a centralized vaccine approach to elicit an immune response that would recognize as many isolates of HIV-1 across various clades. In previous studies, consensus sequences designed for clades B and C gag and envelopes elicited increased breadth of humoral and cellular immune responses [128, 257, 261, 262]. Consensus envelope sequences representing Group M, termed CON-S, elicited antibodies that neutralized multiple envelopes, as well as eliciting cross-clade cellular immune responses [135, 258]. However, viral challenges of CON-S vaccinated monkeys were not reported and therefore the efficacy of the induced immune response elicited by these vaccines is unknown.

Following three intramuscular vaccinations, all monkeys seroconverted by day 14 following the final vaccination. The anti-Env antibody responses detected after vaccination were similar in both vaccine groups. Nonetheless, there were differences in the vaccine efficacy following challenge between the two vaccine groups. Both non-neutralizing and neutralizing antibodies have been implicated in reducing rates of infection by HIV-1 [263, 264]. During the AIDS Vaccine 2011 conference, a report based on the analysis of the sera samples of vaccinated volunteers in the RV144 clinical trial stated that the vaccine elicited antibodies against the V2 region of the HIV-1 envelope were correlated with lower rates of HIV infection [265]. Antisera collected from these vaccinated individuals did not neutralize the infection *in vitro*. Additionally, vaccine induced protection against a neutralization resistant virus in macaques was correlated with antibodies to the V2 region of Env [262]. Whether antibodies that bind to the V2 region are correlated with protection against $SHIV_{SF162p4}$ infection in this study is unclear. There were no antibodies elicited in monkeys vaccinated with Con M or polyvalent consensus Env_{gp140} vaccines that recognized SF162 Env linear peptides, including those specific to V2 (data not shown). Further studies are necessary to determine if the two protected animals in the Con M Env_{gp140} group elicited antibodies recognizing conformational epitopes, such as the V1/V2 scaffold proteins. These epitopes have been used to analyze human sera collected from vaccinated volunteers in the RV144 clinical trial [266]. Determining if antibodies specific to various conformational epitopes on envelope may explain the differences observed in vaccinated animals following SHIV challenge.

Monkeys vaccinated with either polyvalent consensus or Con M Env_{gp140} trimers had neutralizing titers to HIV-1_{SF162}. Neutralizing antibodies against envelope can protect monkeys against viral challenge [244] [240]. However, only one monkey (M4) in the present study had

high neutralizing antibodies (1:320) against $SHIV_{SF162p4}$ that correlated with an undetectable viral titer 14 days after challenge. However, following CD8+ T cell depletion, virus was detected ($<1x10^5$ RNA copies /ml) in the blood indicating that infection was not blocked, but may have been controlled by the vaccine elicited antibodies. T cell responses did not appear to play a role in protecting the monkeys from infection. There was no difference in the number or kinetics in the elicitation of Env or Gag specific IFNγ producing cells following challenge in any of the vaccinated monkeys compared to mock vaccinated animals.

In contrast to monkey M4, no virus was detected in the plasma of monkey M1 even after depletion of CD8+ T cells. Both IFNγ specific T cells and neutralizing antibodies were detected, but it is unclear whether immune responses may have contributed to the protection. Monkey M1 had a Mamu-B*008 MHC class I haplotype, which has been associated with control of SIV_{mac239} virus; the parent virus of the challenge $SHIV_{SF162p4}$ [267]. Therefore, a combination of the neutralizing antibodies, non-neutralizing antibodies and the Mamu-B*008 MHC class I haplotype may have resulted in "sterilizing" protection after viral challenge. However, the Mamu-B*008 MHC class I haplotype was also present in monkey M3, which had similar binding and neutralizing antibody titers as monkey M1, but was not protected from SHIV infection. Even though no viremia was ever detected in monkey M4, it is possible that virus could be located in reservoirs, such as the bone marrow or gut mucosa [268]. The M-T807R1 monoclonal antibody used for CD8+ T cell depletion is specific for cells in the serum and lymph nodes [269], therefore, it may have not depleted cells in reservoirs of hidden virus.

Monkey M4 had neutralizing titers above 1:100 present during pre and post viral challenge, that contributed to reduced viremia and this lends credence to the possibility that a more robust antibody response could have resulted in better protection. Enhancement of the

antibody response could have been accomplished with a better adjuvant. In this study, we used Imject® (Pearce, Rockford, IL, USA) as an adjuvant, but it recently has been shown that Imject® Alum is actually an amorphous aluminum hydroxycarbonate and is distinct from the aluminum-containing adjuvants that are used in licensed vaccines [270]. While this adjuvant is effective at enhancing antibody titers when combined with a vaccine protein, the use of a combination adjuvant *e.g.* TLR agonist and alum may have resulted in a better immune response [271]. The use of AS04 a combination of TLR4 agonist and alum salt induces improved vaccine specific antibodies and B cell responses than alum alone [272]. While Env only vaccines have been successful against homologous challenge, both the human RV144 trial and monkey studies that resulted in significant protection from heterologous challenged, included other HIV protein components [200, 241, 273]. Including Tat in the vaccine formulation induces strong and persistent $CD4^+$ T cells [274] and broadens T cell responses directed against Gag and Env [275, 276]. Gag is known for inducing strong cellular responses that may lead to reduced viral loads [277, 278]. Addition of Gag and/or Tat to our Con M vaccine may have prevented infection or controlled undetectable virus in vaccinated animals more effectively than Con M Env alone.

In conclusion, rhesus macaques were vaccinated with trimerized Env_{gp140} proteins representing consensus sequences for clade A, B, C, E, in a polyvalent mixture or as a single consensus envelope representing the entire Group M. These consensus envelopes elicited antibodies with cross-clade anti-Env binding against a panel of HIV-1 Envs. However, this breadth of antibody binding to HIV-1 Envs did not correlate with the preventing infection by $SHIV_{SF162p4}$.

7.0 SUMMARY AND DISCUSSION

7.1 SUMMARY OVERVIEW

The percentage of the world's population infected with HIV has stabilized over the years [279]. However, the number of people infected with HIV has steadily increased due to new infections, life extending treatments for HIV infection, and the number of new infections outnumbering the number of AIDS-related deaths. Although the disease burden of HIV is disproportionate in developing countries, particularly in southern Africa, the epidemic does not only affect these regions [280, 281]. The District of Columbia in the USA reported more cases of AIDS per 100,000 population in 2006 than were reported in many African countries [282]. In an effort to reduce the HIV global burden, multiple preventative measures are being investigated including education, male circumcision, and developing HIV vaccines. Vaccines have been instrumental in eradicating and reducing the global disease burden of multiple infectious agents such as small pox and polio [283]. Therefore, it is not surprising that the global health sector believes an effective HIV vaccine would be a great tool in the fight against HIV.

Since 1987, many HIV-1 vaccines have been evaluated in monkeys, with over 30 of these vaccines entering human trials either in combination (eg. RV144 human trial combine the AIDSVAX and ALVAC vaccines) or alone [284, 285]. These vaccines had varying degrees of protection in monkeys, however only one human vaccine trial, RV144 (Thailand study), resulted

in significant protection (31%)[69]. The principles guiding the development of an effective HIV vaccine (in addition to safety) is the ability of the vaccine to induce HIV-1 neutralizing antibodies and/or HIV-1 specific CD8+ T cells [286]. Due to the rapid destruction of the immune system upon HIV infection, studies of acute infection have emphasized the need for a protective response to be present before HIV-1 transmission [287, 288].

Studies have reported that antibodies directed at Env are capable of blocking infection and have been linked to reduced acquisition of virus [289]. In addition Env antibodies can also mediate antibody-dependent cellular cytotoxicity (ADCC) by binding to infected cells mediating cytolytic immune responses. These ADCC responses are cross-reactive and therefore can target diverse circulating HIV-1 strains [290, 291]. Other proposed non-neutralizing antibody activity includes complement, and endocytosis and degradation of opsonize HIV viral particles at the mucosal surfaces [292, 293]. Due to its extensive genetic variability and high tolerance for mutation, HIV has a substantial advantage for immune evasion and viral persistence. As a consequence of these properties, significant viral evolution occurs under immune pressure, which has lead to an epidemic fueled by a plethora of genetic variants [206, 294]. The substantial antigenic diversity of HIV-1 combined with the high glycoslation of gp120 and structurally imposed steric constraints of potentially neutralizing epitopes on Env has made developing an effective preventative vaccine a challenge [295-298]. The goal of this project was twofold: first, to develop an HIV-1 envelope vaccine capable of inducing cross-reactive antibody responses prior to infection; and second, to investigate whether the induced antibodies were sufficient to prevent infection in an NHP animal model. Prior to this work being done it was reported that consensus antigens, including Env, increased the breadth of responses when compared to a single primary envelope [105, 299]. In addition, our lab showed that a polyvalent mixture of primary

clade B Envs were able to protect from a clade B SHIV challenge [300]. The studies detailed in this dissertation expanded on the results of the previous work and investigated whether a single group M vaccine would result in protection from the same clade B SHIV. Additionally, the study was designed to induce envelope response prior to challenge, as no envelope responses were observed prior to challenge in the previous work. This is an important difference because of the requirement for circulating antibody during challenge in order to prevent infection, and the presence of detectable envelope responses prior to challenge may aid in identifying a correlate of protection from infection. The results reported in this dissertation did address the goals of the project. Observations from the project demonstrated that both group M consensus Env vaccines (Con M and Polyvalent consensus) elicited pre-challenge antibody titers to a diverse panel of Env proteins from clades A, B and C. In addition two of four animals vaccinated with the Con M Env gp140 resulted in some level of protection, either no infection or reduced viral load after heterologous challenge.

7.2 ANTIGENICITY OF CONSENSUS TRIMERS

The work presented in this dissertation resulted in the development of five consensus envelope immunogens. The modified consensus sequences (A, B, C, E, M) resulted in glycoproteins that formed trimers. The individual gp140 monomers were in a confirmation that allowed for Env trimers to bind to CD4 (HIV envelope receptor) and associate with b12 monoclonal antibody (a well-documented cross-reactive HIV envelope antibody). These results indicated the conservation of functional and antigenic characteristics between the consensus envelopes and wild type HIV envelopes and imply correct structure of the synthetic antigens. The goal of using

the consensus strategy is to capture conserved antigenic epitopes in a population of sequences. Although other consensus envelopes have been shown to retain wild type characteristics, the synthetic nature of the envelope sequences requires that each envelope be evaluated for known antigenic epitopes. The initial investigation into the conserved antigenic epitopes has since expanded into a collaborative study. In the collaborative study, all consensus trimers as well as primary isolates from various years (YU2, R2, ADA, PVO4 and SC42) are being mapped using a collection of mAb including recently isolated quaternary mAb. Quaternary antibodies recognize an epitope formed on the assembled envelope trimer (the quaternary structure of the molecule). A better understanding of the presence or absence of the contact surfaces of these Ab on the various envelopes would aid in correlating the protection outcomes to known conserved epitopes. Also, the results from epitope mapping will improve on the current structure-based vaccine design.

The presence of broadly neutralizing antibodies isolated from infected individuals demonstrates the proof–of–concept that neutralizing antibodies to conserved regions can be elicited. The use of CD4 and mAb b12 to determine conserved antigenic epitopes is important, as the antibodies that bind to these functional epitopes have been shown to neutralize a diverse panel of HIV-1 viruses. For example, mAb b12 has been shown to neutralize up to 75% of B strains and up to 50% of non-B strains [301]. Epitope mapping of prior consensus envelopes was done using broadly neutralizing mAb. However, until recently (2010-11) the only available broadly neutralizing mAbs were isolated from people infected with clade B viruses and some have been shown to be polyreactive (b12, 2G12, 2F, 17b). To expand on the knowledge of the full repertoire of HIV-1 neutralization epitopes, the isolation of new mAbs from non-B infected individuals has been under investigation. These investigations have led to the isolation of new

mAb such as VRCO1, PG9 and PG16. Some of the new antibodies are categorized as quaternary mAb and have been shown to recognize complex epitopes formed by the interactions of the envelope trimer [302, 303].

The epitope mapping of the five consensus envelope trimers constructed in this project included older mAb b12, 17b, and 2G12 as well as more recent quaternary mAb VRCO1, PG9 and PG16. Thus far in the investigation, differences between consensus envelopes were found with mAbs b12, 17b and PG9. Consensus B dissociated at a slower rate from b12 than the other envelopes and was able to bind to 17b prior to CD4 binding. 17b's conserved antigenic epitope is exposed upon CD4 binding [304]. However, in the case of consensus B, Env-CD4 binding was not required for 17b binding (data not shown). These differences indicate that the confirmation of the receptor and co-receptor binding sites of consensus B envelope trimer are different from that present in the other envelopes tested. These differences may have contributed to the lack of protection and limited envelope breadth seen in our lab's previous study where Consensus B VLPs were used for vaccination [300]. In this study, the limited breadth of consensus B was addressed by adding other clade consensus Envs to the vaccine. The breadth of antibodies elicited by the polyvalent consensus envelope vaccine from other clades was increase compared to the consensus B only vaccine. This increase in breadth by combining multiple consensus envelopes is being reported for the first time. However, the proof-of concept of multiple envelopes increasing antibody breadth had been done using multiple primary envelopes [257, 305]. The work reported in this dissertation has provided evidence that the same outcome could be achieved by a mixture of clade consensus envelopes, eliminating the issue of determining which primary envelopes to use in the polyvalent vaccine. Instead of one envelope

isolate being chosen, all candidate envelope isolates could be used to determine a consensus clade envelope to then use in a polyvalent vaccine.

Epitope mapping of the consensus envelopes with recently identified and characterized quaternary monoclonal antibodies (PG9) [306-308] have also demonstrated differences between the envelopes. These antibodies were first isolated from individuals infected with viruses from clade A. PG9 has been shown to bind to glycans associated with the V1/V2 region of Env gp120 [307] and antibodies elicited in the V1/V2 region of Env have been implicated as a correlate of reduced infection in the RV144 Thailand study [69]. The PG9 monoclonal antibody binds to Consensus M and A trimers only. The PG9 antibody was isolated from an individual infected with a clade A virus [309]. Therefore, it is not surprising that the consensus A envelope was recognized by the antibody. The binding of PG9 to HIV-1 envelopes is influenced by subtle differences in loop length, spatial orientation of glycan residues and the net charge of β sheet C region that directly binds to PG9 CDRH3 within V2 loop. In initial screening of the consensus envelopes there was no significant difference in the length of the V2 loop and glycosylation sites. However, spatial orientation of glycan residues may play a role in the absence of PG9 binding to consensus Envs B, C, and E. The inclusion of clade A sequences into the consensus M resulted in the inclusion of PG9 binding site. The amount of PG9 epitope present in the polyvalent vaccine is at least 25% less than its presence in the Consensus M vaccine. The binding of PG9 to consensus A and M provides evidence that these envelopes could elicit similar antibodies. The differences in the amount/concentration of the PG9 epitope presence in the two vaccines may explain the difference observed after challenge between the two groups.

Identifying where the vaccine elicited antibodies bound to the antigen would add to the information already gathered from this project about the epitopes, structure and protective

efficacy of antibodies. Experiments could be designed to isolate cross-reactive mAbs from vaccinated NHP and would then be used to identify their antigenic epitopes. This would address the question of whether different antibodies to different epitopes were elicited by the two vaccines. Given the differences being observed in the epitope mapping study and the immunological results in this project, differences would be expected. Two possible scenarios that may be identified 1) Same antibodies in all vaccinated animals, but protected animals have a higher concentration of antibodies that bind to conserve epitopes (such as CD4 binding site) compared to unprotected animals 2) Differences in antibodies: protected vaccinated animals have antibodies that bound to multiple epitopes compared to unprotected animals. In a natural infection, the ability to induce a protective response against a virus is due to polyclonal antibodies. These polyclonal antibodies directed at multiple different epitopes act in synergy to inactivate the virus. In addition, the project may discover yet unknown viral epitopes targeted by cross-reactive antibodies which would lead to a better understanding of current structure based design.

7.3 IMMUNOGENICITY AND EFFICACY

It is well known that antigenicity and immunogenicity are not inter-changeable, therefore it was necessary to evaluate the immunogenicity of consensus envelopes. Animal vaccination with the consensus envelopes induced antibodies able to detect multiple envelopes from clades A, B, C and E. Non-human primate vaccination also resulted in detectable neutralizing antibodies to the challenge envelope SF162 *in vitro*. The induction of cross-reactive antibodies has been shown with Consensus M in small animals and with polyvalent primary envelopes. This

is the first time that this envelope cross-reactivity has been shown using a polyvalent consensus Env only vaccine. Furthermore, the broadly reactive antibody response was observed in both small animal and NHP models. The study directly compared a single group M envelope vaccine and a polyvalent group M vaccine and showed that there was no significant difference in the breadth of the elicited response. By comparing a group M consensus Env to a polyvalent mixture of consensus Env antigens, we stringently evaluated the breadth of vaccine-induced antibody responses. Previous studies have only compared a consensus group M Env to a single primary envelope in the same study and reported greater antibody and T cell breadth with consensus group M Env. The breadth of a mixture of 5 Env isolates from different clades (A, B, C) and a single Gag was also investigated and shown to be superior to the use of a single Env isolate [305]. Additionally, our finding that a polyvalent mixture did not improve antibody breadth is noteworthy because a single immunogen (group M) would be easier and more economical to manufacture. However, a major pitfall of this study was the limitation of the neutralization breadth.

Neutralizing antibodies are known to completely block HIV in animal model [310], maternal NAb aid in protection against perinatal transmission [311, 312] and evidence has suggest that they might protect from superinfection [313]. The presence of broadly neutralizing antibodies in infected individuals is infrequent [314, 315] and studies of their structure and phenotype of these antibodies demonstrate unusual features. These unusual features and low frequency suggest that the mAb arose from atypical B-cell induction pathways. For example, 2F5 and 4E10 have been reported to be self-reactive, and b12 has an unusually long third complementarity determining region of antibody heavy chains (CDRH3) for a mAb [316, 317]. This CDRH3 allows b12 to overcome the steric hindrance that protects the envelope's receptor

binding pocket from neutralizing antibodies [317]. These observations have brought to realization that understanding envelope antibody affinity maturation may have a greater impact on vaccine design [318]. It is known that broadly neutralizing mAbs demonstrate moderate to high levels of affinity maturation and may require repeated antigen stimulation. This need for constant antigen stimulation may be why broadly neutralizing mAbs occur in patients who are infected for several years or more. Strategies which could be used to facilitate antibody affinity maturation include 1) use of adjuvants that lead to the induction of cytokines for better germinal centers, and 2) multiple boosts or alternate platforms that provide antigen stimulation for prolonged periods.

In this project, the adjuvant Imject® alum was used to help with the induction of cross-reactive antibodies. This adjuvant was successful when used in another study performed in the lab using a consensus influenza envelope vaccine COBRA [209]. The limited success (low IgG titers and limited neutralizing breadth) in this project could be attributed to the recent findings that Imject® Alum is actually an amorphous aluminum hydroxycarbonate and is distinct from the aluminum-containing adjuvants that are used in licensed vaccines [319]. Imject® Alum formulation of amorphous aluminum hydroxycarbonate and crystalline magnesium hydroxide is similar that that seen in Maalox antacid. Such chemical differences might be critical to the outcome of the immune response [320]. Licensed alum has been shown to activate NLRP3 inflammasome. Several particulate materials activate the NLRP3 inflammasome in a similar way, and physico-chemical properties are key. For example, although Monosodium urate or uric acid (MSU) crystals (the etiological agent of gout) activate NLRP3, crystals of its analogue allopurinol do not. Therefore, although it has been shown that Imject® Alum is able to activate caspase-1 *in vitro* as much as licensed aluminium adjuvants do [321] the *in vivo* effects of a

formulation with different physico-chemical parameters are unpredictable and could partially account for the low level of immunogenicity seen in monkeys. Therefore the use of another adjuvant(s) may have resulted in a more robust and higher affinity antibody response. Inducing antibodies with greater affinity and higher titers, based on the knowledge from the isolated broadly NAb, would lead to more effective antibodies. The use of multiple adjuvants targeting different aspects of the innate system has led to higher antigen specific antibodies compared to the use of one adjuvant [322].

In addition to adjuvants as mentioned above, persistent antigen may play a role in the development of broadly neutralizing antibodies. This persistent antigen exposure has been implicated in the protective efficacy of the live attenuated vaccine [323-325], however the vaccine is not safe. DNA launched vectors are based on Flaviviruses, mainly the Venezuelan equine encephalitis (VEE) virus and the Sindbis virus, and the platforms allows the viral replicon (no structural genes) to be launched from a plasmid [326, 327]. The vector system retains the feature of high antigen presentation without the necessity of structural proteins [328]. These platforms are currently being investigated to eliminate induction of antibodies to the viral vectors structural proteins [329]. Our lab has partnered with Dr. Klimstra to test the DNA launch system with our HIV vaccine immunogens. The DNA launch plasmid is based on VEE and is under the cytomegalovirus immediate early promoter. An initial test of the launch vector showed that the vector expressed GFP at the injection site longer than vaccination with replicons of the same virus (unpublished data). It has been shown in literature that the use of VEE launch vector expressing an HIV envelope results in: 1) high level of protein, 2) type I IFN in vitro (induces Th-1 responses in mice), 3) effective prime to a protein boost, 4) increase humoral and cellular responses over VEE DNA only, 5) does not induce VEE replicon antibodies [327]. Preliminary

mouse studies with a Consensus M DNA launch vector resulted in a continuous increase in anti-envelope antibody titer responses after the second vaccination Figure 23 (unpublished data). A study using the DNA launch system expressing Consensus M will be used in a vaccine regimen to answer the question “Does persistent antigen presentation in a vaccine regimen increase the breadth and affinity of neutralizing antibodies elicited by the consensus envelope.”

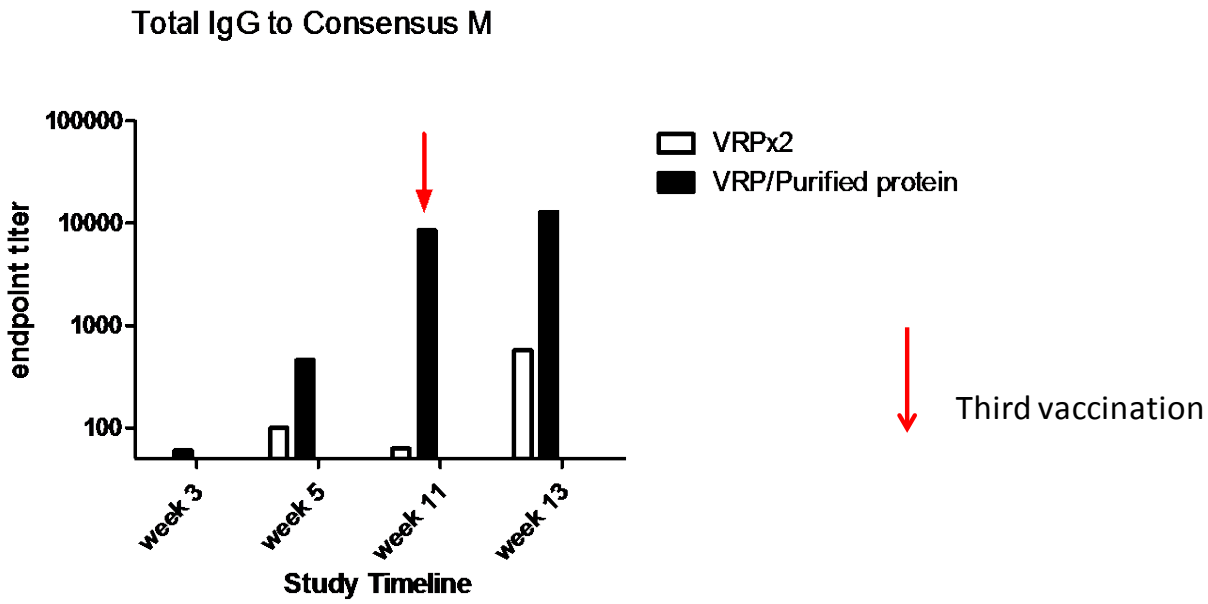


Figure 23: DNA launch Consensus M Immunogenicity

BalB/c mice were vaccinated at weeks 0,3 and 11. Sera were collected at weeks 3,5,11 and 13 post vaccination. The animal groups were 1) VEE DNA launch x2 /purified protein trimer boost (10µg) and VEE DNA launch prime/2x purified protein trimer boost (10µg). Total IgG responses to homologous envelope were determined via ELISA to consensus M. The endpoint titer is described on the y–axis and the weeks post vaccination on the x-axis.

The results in this project have provided additional evidence to support the use of consensus envelopes to increase the breadth of envelope specific humoral responses. This humoral binding breadth and limited neutralizing breadth did not result in protection from heterologous challenge. Although vaccination with both vaccines elicited binding and neutralizing antibodies to the challenge envelope, only two animals in the Consensus M group had no detectable viral RNA in the presence of an "intact" immune system. Upon CD8 T cell depletion, detectable levels of viral RNA were observed in the plasma of one of the animals, referred to as M4. This depletion antibody used has only been reported to deplete CD8 T cells from blood and lymph nodes [330, 331]. Monkey M4 was not protected from viral infection as indicated by the emergence of virus after depletion. The humoral response elicited by vaccination of monkey M4 resulted in a reduction in the virus inoculum. The antibody present together with the T cell response detected after challenge were sufficient to suppress viral loads to less than 50 RNA copies/ml of plasma. The other CD8 T cells depleted monkey (M1) had no detectable virus in its plasma even after CD8 T cell depletion. However, this monkey was exposed to virus sufficient to induce IFN-γ responses detectable by ELISPOT. Therefore, it is likely that the animal was able to clear the virus from the blood. Complete viral clearance from other tissues, such as the gut, cannot be confirmed by CD8 T cell depletion as the antibody used has only been reported to deplete CD8 T cells in blood and lymph nodes. The other explanation of M1 protection is that the monkey received a reduced dose of infectious virus compared to the other animals upon challenge allowing the vaccine elicited response to be sufficient to prevent infection.

These results bring into question how much antibody is needed to prevent infection. Experiments using low dose mucosal challenge models show that the level of antibody required

to protect from infection may be 10 fold lower than previously thought [332, 333]. Serum levels of antibodies required in these studies vary based on the epitope target and mechanism (Neutralizing or Fc-mediated effector) of the antibody. An example of epitope target determining antibody level needed was observed when monkeys were protected with lower serum levels of the broadly neutralizing antibody 2G12, which recognize a cluster of high-mannose glycans on gp120, then serum levels needed with b12 which binds to the CD4 epitope [332, 333]. This observation suggests that some antigenic epitopes induced more effective neutralizing antibodies than others. This difference in the level of antibodies needed to prevent infection being dependent on epitope target may explain the differences seen after challenge. In this study the level of total IgG was similar in all vaccinated animals, however not all animals were protected from infection (quality vs. quantity). While non-human primate data suggests a strong correlation between neutralizing antibodies (NAb) and protection, the Fc-mediated effector functions (ADCVI, ADCC) of antibodies have also been shown to have a role in protection [334, 335]. Finally, serum levels required for protection may vary depending on the viral challenge.

Based on results from this project and information in the field, how much antibody is needed for protection is a delicate balance between the type of antibody present and the viral dose expose to the animal/ subject. In addition, if the antibodies present are not sufficient to prevent infection, the presence of an effective T cell response at the site of viral challenge is required to clear all infected cells before spread. Figure 24 below shows a model of how the balance between antibody and viral challenge resulted in multiple outcomes after challenge of the vaccinated animals in this study.

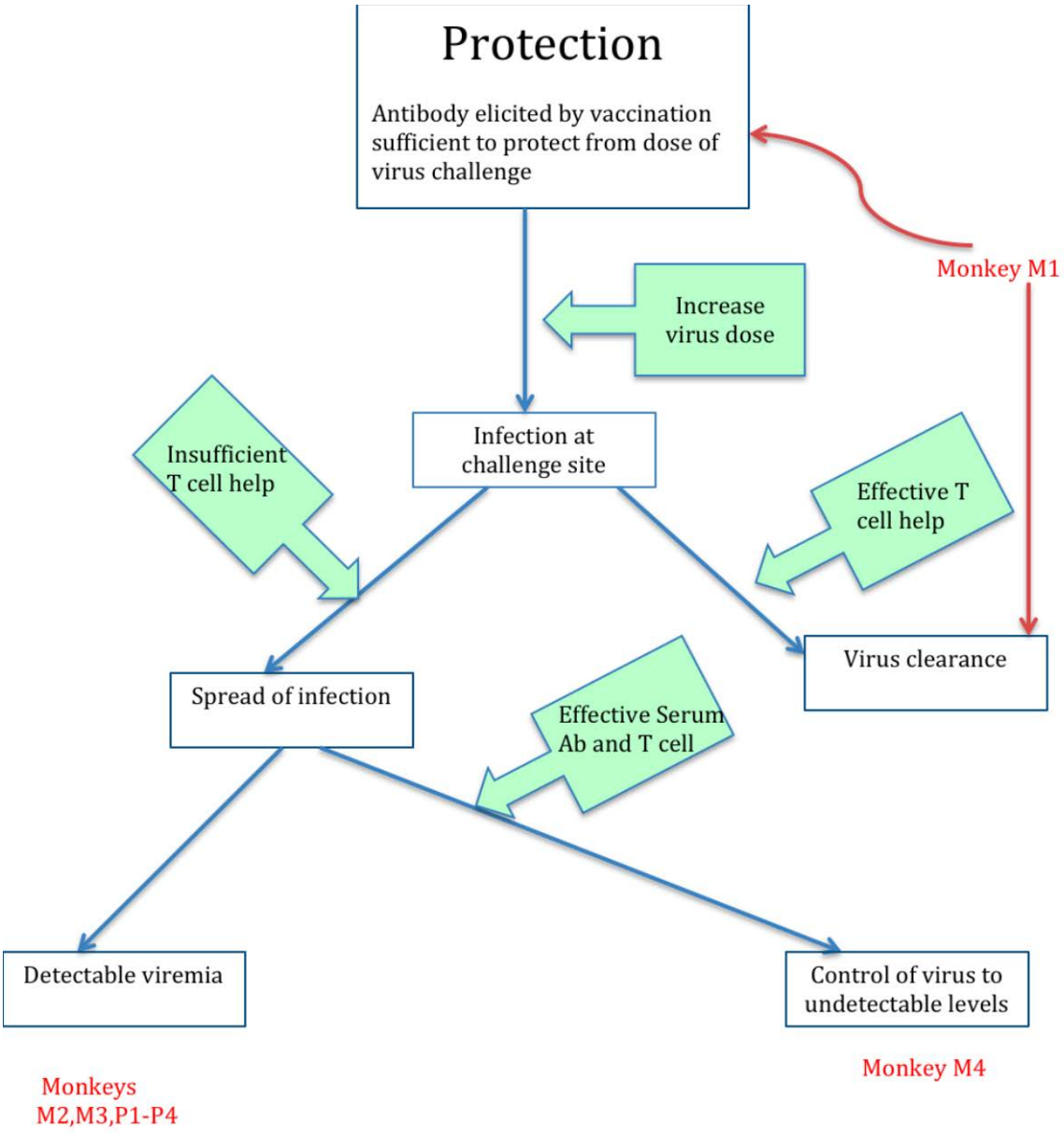


Figure 24: Model of the outcome of animals in this study after challenge.

Monkey M1: was protected from challenge either due to sufficient antibodies present to protect from the viral dose given or the antibodies reduced the infectious dose enough to allow T cell present at the mucosal site to clear infected cells. Monkey M4: the antibodies and T cells present at the time of challenge was insufficient to prevent infection and spread of virus. However, the serum antibody and T cell responses were sufficient to reduce virus to undetectable serum levels. All other vaccinated animals did not have a sufficient antibody response to protect from viral challenge; either by preventing infection or reducing viral loads that would reduce viral transmission**.**

7.4 FUTURE STUDIES

The next NHP vaccine study should consist of a vaccine regimen that includes an adjuvant system (more than one type of adjuvant) and the DNA launch vectors with not only Consensus M envelope but T cell antigens such as Tat and Gag. It was shown that the consensus envelope elicits binding breadth to multiple clades A, B, and C. Therefore, with better antibody affinity maturity the breadth and strength of the cross-clade neutralizing antibodies would increase. The addition of T cell antigens would elicit $CD4^+$ T cell help that aid with the antibody maturity. Germinal center (GCs) formation is essential for the development of high-affinity antibody-secreting plasma cells and long-lived memory B cells generation [336]; a process that is highly dependent on CD4 T-cell help [337]. Apart from $CD4^+$T help the presence of effective $CD8^+$T would be instrumental in the clearance of infected cells when sterilizing protection is not attained by the immune response elicited by vaccination. In terms of challenge, the multiple low dose challenges model should be used as it is more "real world" than the one high dose challenge. Furthermore, a more relevant SHIV should be used: the envelope SF162 was isolated from a chronic infection and, until recently, was the better of two SHIVs available for mucosal challenge. However, from late 2011 other groups have described additional SHIVs capable of infection at the mucosal surface with more relevant envelopes. These SHIVs have envelopes that were isolated early in infection and may be a better representation of viruses responsible for transmission [338-340].

This project did not give rise to a broadly reactive HIV-1 vaccine that resulted in significant protection after SHIV challenge; however contributions have been made to the field. In summary, five consensus *env* trimers were designed and constructed and successfully expressed as trimeric envelope proteins. These synthetic envelopes retained functional and

antigenic epitopes, supporting previous work in the field reporting that synthetic consensus envelopes are similar to envelopes of isolated HIV viruses. Also, the five consensus Env trimers are now available as tools in mapping studies aimed at correlating presence of antigenic epitopes to induction of similar known antibodies and their role in protection from virus challenged.

The Env trimers were also immunogenic in mice and monkeys. The antibodies elicited were able to bind to multiple envelopes from clades A, B, C and neutralize the challenge virus envelope SF162 via the *in vitro* neutralizing TZM-bl assay. This was the first study that compared to group M vaccines directly: a single consensus M vaccine versus a polyvalent mixture of clade consensus envelopes. The mouse studies showed that all envelopes used in the polyvalent mixture were immunogenic on their own and this immunogenicity was not dampened when envelopes were placed into one vaccine. The results described in this dissertation have proven that a single group M envelope is able to induce similar breadth as a mixture of envelopes from multiple clades that group M comprises.

Previous heterologous SHIV challenges reported in the literature have all been clade specific, most comprised of other HIV proteins mainly Gag and Tat and challenged with a multiple low dose infection model. In addition, the protection seen in those studies was not 100%. This is the first study where a designed Group M Env only vaccine was used to protect from SHIV challenge. The protection from infection (M1) and reduced viral burden (M4) of 2 of 4 animals vaccinated with the Con M Envgp140 shows a trend to protection of the single consensus envelope. This protection could be increased with a vaccine regimen that would result in a more robust and higher affinity antibody response. This finding is important as it supports the use of a group M vaccine being capable of providing some protection (prevention of infection or reduced viral burden) from virus challenge.

APPENDIX A

CONSENSUS C VACCINE STUDY IN NHP

Shown below is the data from a study performed at the Puerto Rico Primate facility to investigate the immunogenicity and efficacy of the consensus C envelope in the NHP model. The monkeys were given 2xDNA vaccinations *(encoding gp140 consensus C trimers)* 1 mg each *(500µg IM& 500µg ID)* followed by 2xSHIV Con C VLP protein *(500ug).*VLP vaccination were given formulated with 125µg of CpG in a total volume of 500µl. Eight weeks after final vaccination the animals were challenged with SHIV1157ipd3N4 repeated low dose 25TCID50.

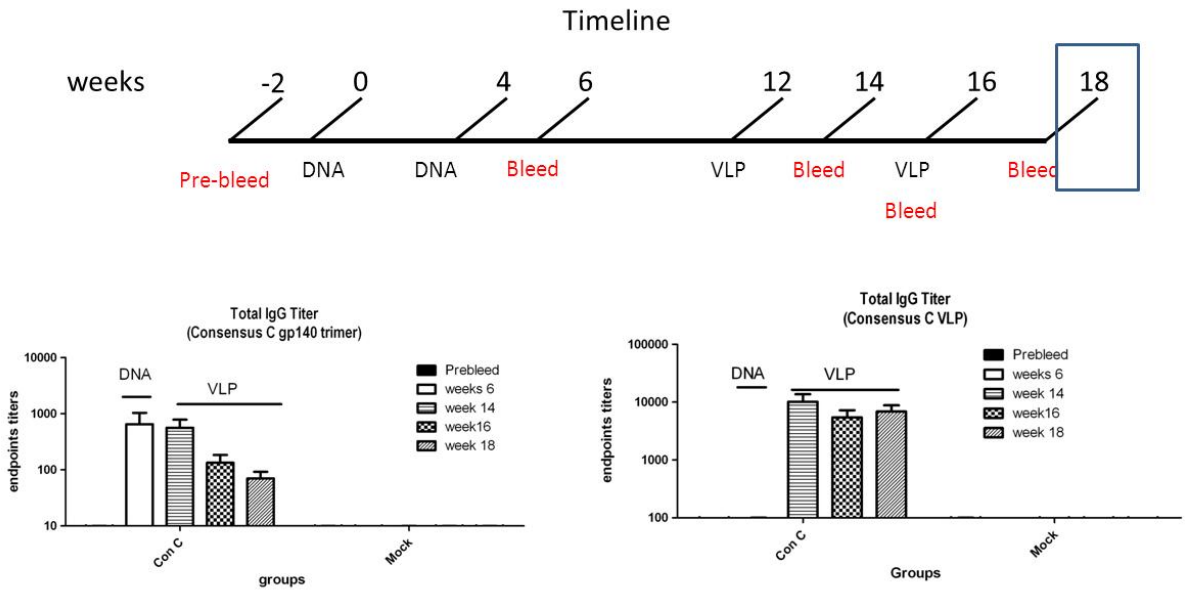


Figure 25: Immunogenicity of Consensus C vaccine

NHP were vaccinated at weeks 0, 4, 12 and 16 with blood collected 14 days after each vaccination. Vaccination consisted of 2xDNA vaccinations (encoding gp140 consensus C trimers) 1 mg each (500µg IM& 500µg ID) followed by 2xSHIV Con C VLP protein (500µg) formulated with 125µg of CpG. Sera collected at week 18 were used to determine total IgG via ELISA for each vaccine group against consensus C vaccine. The endpoint titer is described on the y–axis and the animal groups described on the x-axis.

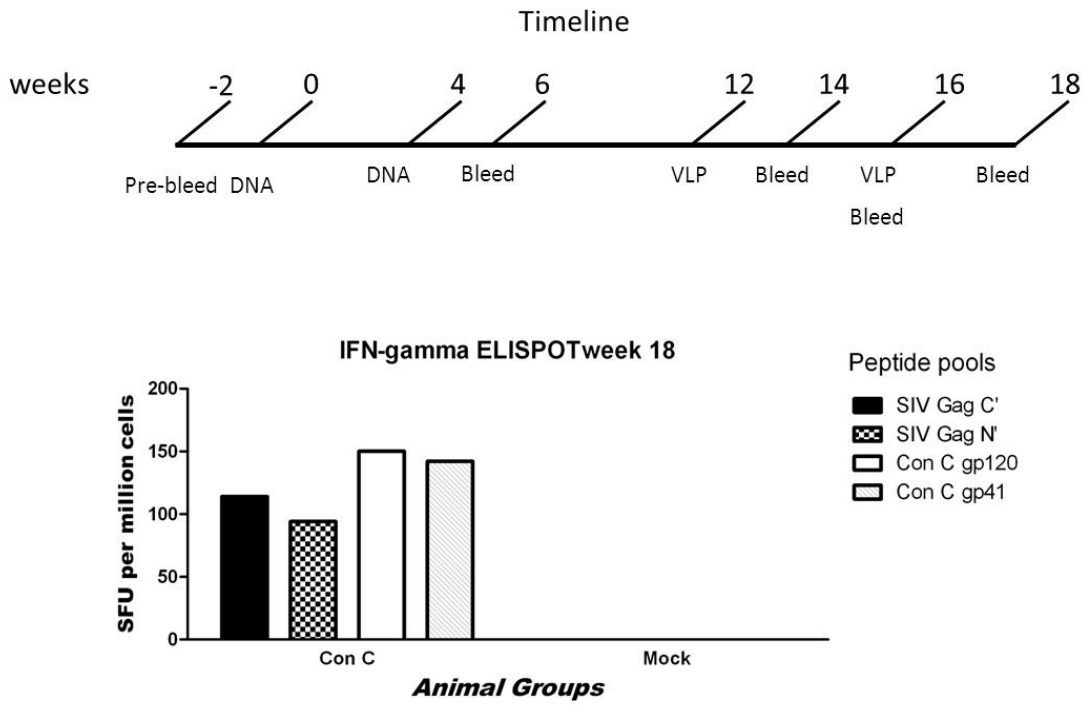
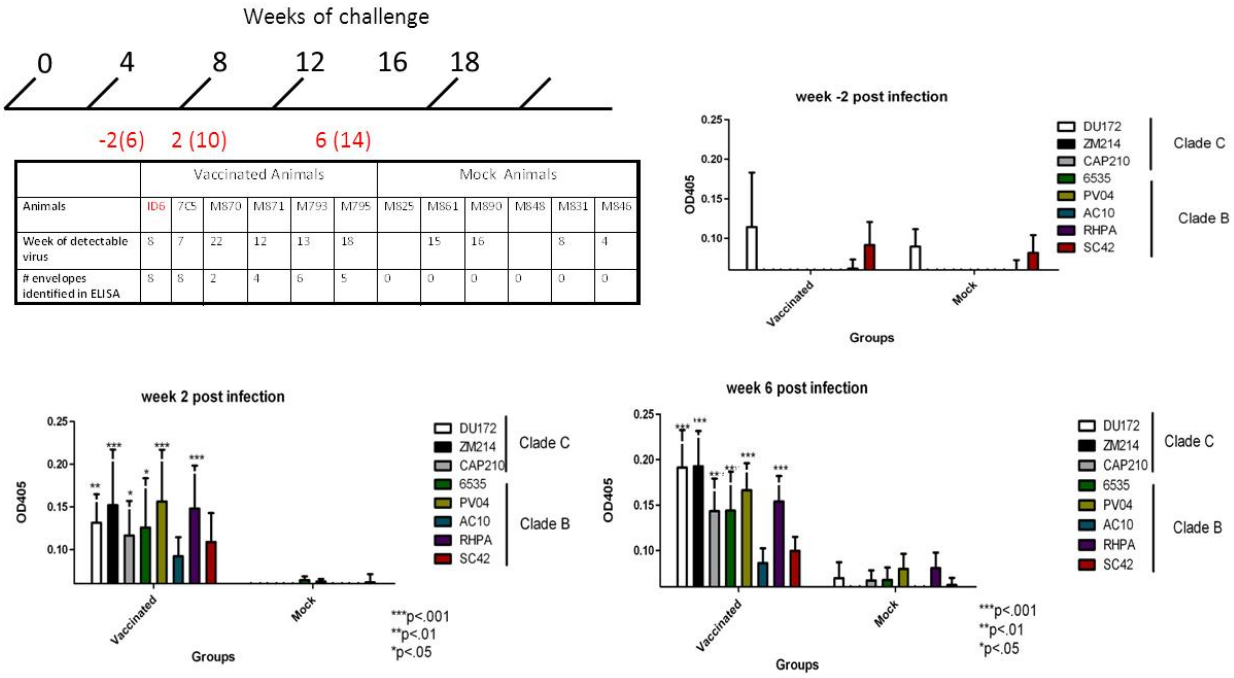


Figure 26: IFN-γ ELISPOT Responses after Vaccination

INF-γ ELISPOTs were performed using monkey PBMCs collected week 18 (2 weeks after last vaccination) $1x10^5$ PBMCs were stimulated with 30µg of Gag peptide pools representing SIV_{mac239} or Env pools representing Consensus C (overlapping peptides, 15-mers with11 amino acid overlap NIH AIDS Research and Reference Reagent program. Spot forming units (SFU) per one million PBMCs are listed on the y-axis and animal groups listed on the x-axis.



	Vaccinated Animals						Mock Animals					
Animals	ID6	7C5	M870	M871	M793	M795	M825	M861	M890	M848	M831	M846
Week of detectable virus	8	7	22	12	13	18		15	16		8	4
# envelopes identified in ELISA	8	8	2	4	6	5	0	0	0	0	0	0

Figure 27: Kinetics of Cross-binding Envelope responses

Anti-Env IgG was detected in the serum samples (1:100 dilution) against a panel of primary Env_{gp120s} from clades B ,and C via ELISA. Bar values represent the geometric mean titer (95% confidence interval) at an OD450. The OD450 values are displayed on the y-axis and animal groups are listed on the x-axis. A two-way ANOVA with Bonferroni's post-test was used to evaluate Statistical significance between the vaccines for each test antigen. A *p*-value of less than 0.05 was considered significant.

Table 6: Neutralizing Antibody responses to challenge virus envelope

Neutralization of HIV $_{SHIV1157ipd3N4}$ by serum collected post-challenge. Neutralizing antibodies to challenge virus after infection were detected sooner. Increase envelope breath seen in the vaccinated animals vs. mocks at week 2 post detectable virus

Envelope Identity	clade	% Identity to Consensus C
DU172	C	88
ZM214	C	86
CAP210	C	85
6535	B	77
PVO4	B	77
AC10	B	76
RHPA	B	78
SC42	B	78

	Vaccinated Animals						Mock Animals					
Animals	ID6	7C5	M870	M871	M793	M795	M825	M861	M890	M848	M831	M846
Week of detectable virus	8	7	22	12	13	18		15	16		8	4
# envelopes identified in ELISA	8	8	2	4	6	5	0	0	0	0	0	0
Week of detectable neuts after detectable virus	1	1		2	2						6	5

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BIBLIOGRAPHY

Literature Cited

1. Bower, J.F., et al., *HIV-1 Envgp140 trimers elicit neutralizing antibodies without efficient induction of conformational antibodies.* Vaccine, 2006. **24**(26): p. 5442-5451.
2. Yang, X., et al., *Highly Stable Trimers Formed by Human Immunodeficiency Virus Type 1 Envelope Glycoproteins Fused with the Trimeric Motif of T4 Bacteriophage Fibritin*, in *Journal of Virology*2002. p. 4634-4642.
3. Barre-Sinoussi, F., et al., *Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-871.
4. Gallo, R.C., et al., *Isolation of Human T-Cell Leukemia Virus in Acquired Immune Deficiency Syndrome (AIDS).* Science, 1983. **220**(4599): p. 865-867.
5. World Health Organization, U., *Global summary of the AIDS epidemic 2010*, 2011, World Health Organization.
6. Soares, M.A., R.M. Brindeiro, and A. Tanuri, *Primary HIV-1 drug resistance in Brazil*, 2004. p. S9-S13.
7. Vitoria, M., et al., *The Global Fight Against HIV/AIDS, Tuberculosis, and Malaria*, 2009. p. 844-848.
8. Bos, J.M. and M.J. Postma, *The Economics of HIV Vaccines: Projecting the Impact of HIV Vaccination of Infants in Sub-Saharan Africa*, 2001. p. 937-946.
9. Cockcroft, A., *Global impact of AIDS on work*, 2002. p. 280-284.
10. Gayle, H.D. and G.L. Hill, *Global Impact of Human Immunodeficiency Virus and AIDS*, 2001. p. 327-335.
11. Gazzard, B., *Tuberculosis, HIV and the developing world.* Clinical Medicine, Journal of the Royal College of Physicians, 2001. **1**(1): p. 62-68.
12. Williams, B.G., et al., *The impact of HIV/AIDS on the control of tuberculosis in India*, 2005. p. 9619-9624.
13. Zumla, A., et al., *Impact of HIV infection on tuberculosis*, 2000. p. 259-268.
14. RAFAEL NAJERA, M.M.H., PhD, and R. de ANDRES, MSc, Madrid, *Human Immunodeficiency Virus and Related Retroviruses.* West J Med., 1987. **147**(6): p. 702-8.
15. Clavel, F., et al., *Isolation of a new human retrovirus from West African patients with AIDS*, 1986. p. 343-346.
16. Minassian, A.A., et al., *Monoclonal antibodies against human immunodeficiency virus (HIV) type 2 core proteins: cross-reactivity with HIV type 1 and simian immunodeficiency virus*, 1988. p. 6939-6943.

17. D.L. Robertson, J.P.A., J.A. Bradac, J.K. Carr, B. Foley, R.K. Funkhouser, F. Gao, B.H. Hahn, M.L. Kalish, C. Kuiken, G.H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P. M. Sharp,S. Wolinsky, and B. Korber, *HIV-1 Nomenclature Proposal:A Reference Guide to HIV-1 Classification*, 199, HIV Databases Review Article.
18. Plantier, J.-C., et al., *A new human immunodeficiency virus derived from gorillas.* Nat Med, 2009. **15**(8): p. 871-872.
19. Nkengasong, J.N., et al., *Genotypic subtypes of HIV-1 in Cameroon.* AIDS, 1994. **8**(10): p. 1405-1412.
20. W. JANSSENS, L.H., K. FRANSEN, M. TEMMERMAN, A. LEONAERS, T. IVENS, J. MOTTE, P. PIOT, and G. VAN DER GROEN., *Genetic Variability of HIV Type 1 in Kenya* AIDS Research and Human Retroviruses, 1994. **10**(11): p. 1577-1579.
21. Gharu, L., R. Ringe, and J. Bhattacharya, *Evidence of extended alternate coreceptor usage by HIV-1 clade C envelope obtained from an Indian patient.* Virus Research, 2012. **163**(1): p. 410-414.
22. Yang, J., et al., *A natural theaflavins preparation inhibits HIV-1 infection by targeting the entry step: Potential applications for preventing HIV-1 infection.* Fitoterapia, 2012. **83**(2): p. 348-355.
23. Checkley, M.A., B.G. Luttge, and E.O. Freed, *HIV-1 Envelope Glycoprotein Biosynthesis, Trafficking, and Incorporation.* Journal of Molecular Biology, 2011. **410**(4): p. 582-608.
24. Gu, M., J. Rappapor, and S.H. Leppla, *Furin is important but not essential for the proteolytic maturation of gp160 of HIV-1*. FEBS Letters, 1995. **365**(1): p. 95-97.
25. de la Hera, M.G., et al., *Gender differences in progression to AIDS and death from HIV seroconversion in a cohort of injecting dug users from 1986 to 2001*, 2004. p. 944-950.
26. Henrard, D.R., et al., *Natural History of HIV-1 Cell-Free Viremia*, 1995. p. 554-558.
27. Chandra, P., et al., *Quality of life in HIV subtype C infection among asymptomatic subjects and its association with CD4 counts and viral loads – a study from South India.* Quality of Life Research, 2006. **15**(10): p. 1597-1605.
28. Fraser, C., et al., *Variation in HIV-1 set-point viral load: Epidemiological analysis and an evolutionary hypothesis*, 2007. p. 17441-17446.
29. Mitsuya, H., et al., *3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro.* Proceedings of the National Academy of Sciences, 1985. **82**(20): p. 7096-7100.
30. von Kleist, M., et al., *HIV Quasispecies Dynamics during Pro-Active Treatment Switching: Impact on Multi-Drug Resistance and Resistance Archiving in Latent Reservoirs.* PLoS ONE, 2011. **6**(3): p. e18204.
31. Erik, D.C., *Antiretroviral drugs.* Current Opinion in Pharmacology, 2010. **10**(5): p. 507-515.
32. De Clercq, E., *Antiretroviral drugs.* Current Opinion in Pharmacology, 2010. **10**(5): p. 507-515.
33. Gulick, R.M., et al., *Treatment with Indinavir, Zidovudine, and Lamivudine in Adults with Human Immunodeficiency Virus Infection and Prior Antiretroviral Therapy.* New England Journal of Medicine, 1997. **337**(11): p. 734-739.

34. Crabtree-Ramirez, B., et al., *Effectiveness of Highly Active Antiretroviral Therapy (HAART) Among HIV-Infected Patients in Mexico.* AIDS Research and Human Retroviruses, 2010. **26**(4): p. 373-378.
35. Greenbaum, A.H., et al., *Effect of age and HAART regimen on clinical response in an urban cohort of HIV-infected individuals.* AIDS, 2008. **22**(17): p. 2331-2339 10.1097/QAD.0b013e32831883f9.
36. Von Kleist, M., et al., *HIV Quasispecies Dynamics during Pro-Active Treatment Switching: Impact on Multi-Drug Resistance and Resistance Archiving in Latent Reservoirs.* PLoS ONE, 2011. **6**(3): p. e18204.
37. Paci, P., et al., *Timely HAART initiation may pave the way for a better viral control.* BMC Infectious Diseases, 2011. **11**(1): p. 56.
38. Kronenberg, A., H.M. Riehle, and H.F. Gunthard, *Liver failure after long-term nucleoside antiretroviral therapy.* The Lancet, 2001. **358**(9283): p. 759-760.
39. John, M., et al., *Chronic hyperlactatemia in HIV-infected patients taking antiretroviral therapy.* AIDS, 2001. **15**(6): p. 717-723.
40. World Health Organization, W., *ANTIRETROVIRAL THERAPY FOR HIV INFECTION IN ADULTS AND ADOLESCENTS: Recommendations for a public health approach* W.H.A. Programme, Editor 2006, World Health Organization p. 1-134.
41. Letang, E., et al., *Incidence and Predictors of Immune Reconstitution Inflammatory Syndrome in a Rural Area of Mozambique.* PLoS ONE, 2011. **6**(2): p. e16946.
42. Soneja, M. and S. Sharma, *HIV & immune reconstitution inflammatory syndrome (IRIS).* Vol. 134. 2011. 866-877.
43. Tappuni, A.R., *Immune Reconstitution Inflammatory Syndrome.* Advances in Dental Research, 2011. **23**(1): p. 90-96.
44. Lin, J.-N., et al., *Immune reconstitution inflammatory syndrome presenting as chylothorax in a patient with HIV and Mycobacterium tuberculosis coinfection: a case report.* BMC Infectious Diseases, 2010. **10**(1): p. 321.
45. Broder, S., *The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic.* Antiviral Research, 2010. **85**(1): p. 1-18.
46. Montagnier, L., *25†years after HIV discovery: Prospects for cure and vaccine.* Virology, 2010. **397**(2): p. 248-254.
47. Aaron N. Endsley, N.N.S., Rodney J.Y. Ho, *Combining Drug and Immune Therapy: A Potential Solution to Drug Resistance and Challenges of HIV Vaccines?* Current HIV Research, 2008. **6**: p. 401-410.
48. Monteiro, J.P., et al., *Genetic variability of human immunodeficiency virus-1 in Bahia state, Northeast, Brazil: High diversity of HIV genotypes.* Journal of Medical Virology, 2009. **81**(3): p. 391-399.
49. Cuevas, M.T., et al., *Short Communication: Biological and Genetic Characterization of HIV Type 1 Subtype B and Nonsubtype B Transmitted Viruses: Usefulness for Vaccine Candidate Assessment.* AIDS Research and Human Retroviruses, 2010. **26**(9): p. 1019-1025.
50. Robertson, D.L., et al., *HIV-1 Nomenclature Proposal.* Science, 2000. **288**(5463): p. 55.
51. Brown, R.J.P., et al., *Inter-compartment recombination of HIV-1 contributes to env intra-host diversity and modulates viral tropism and senstivity to entry inhibitors.* J. Virol., 2011: p. JVI.00131-11.

52. Delelis, O., et al., *Integrase and integration: biochemical activities of HIV-1 integrase.* Retrovirology, 2008. **5**(1): p. 114.
53. Engelman, A., et al., *Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication.* J. Virol., 1995. **69**(5): p. 2729-2736.
54. Miedema, F., *A brief history of HIV vaccine research: stepping back to the drawing board?* AIDS, 2008. **22**(14): p. 1699-1703.
55. Virgin, H.W. and B.D. Walker, *Immunology and the elusive AIDS vaccine.* Nature, 2010. **464**(7286): p. 224-231.
56. Finzi, D., et al., *Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy.* Nat Med, 1999. **5**(5): p. 512-517.
57. Carter, C.C., et al., *HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs.* Nat Med, 2010. **16**(4): p. 446-451.
58. Chun, T.-W., et al., *Persistence of HIV in Gut-Associated Lymphoid Tissue despite Long-Term Antiretroviral Therapy.* Journal of Infectious Diseases, 2008. **197**(5): p. 714-720.
59. Lerner, P., et al., *The Gut Mucosal Viral Reservoir in HIV-Infected Patients Is Not the Major Source of Rebound Plasma Viremia following Interruption of Highly Active Antiretroviral Therapy.* J. Virol., 2011. **85**(10): p. 4772-4782.
60. Wong, J.K., et al., *Recovery of Replication-Competent HIV Despite Prolonged Suppression of Plasma Viremia.* Science, 1997. **278**(5341): p. 1291-1295.
61. Siliciano, J.D. and R.F. Siliciano, *A long-term latent reservoir for HIV-1: discovery and clinical implications.* Journal of Antimicrobial Chemotherapy, 2004. **54**(1): p. 6-9.
62. Emini, E.A., et al., *Prevention of HIV-1 infection in chimpanzees by gpl20 V3 domain-specific monoclonal antibody.* Nature, 1992. **355**(6362): p. 728-730.
63. Putkonen, P., et al., *Prevention of HIV-2 and SIVsm infection by passive immunization in cynomolgus monkeys.* Nature, 1991. **352**(6334): p. 436-438.
64. Prince, A.M., et al., *Prevention of HIV Infection by Passive Immunization with HIV Immunoglobulin.* AIDS Research and Human Retroviruses, 1991. **7**(12): p. 971-973.
65. *HIV gp120 Vaccine - VaxGen: AIDSVAX(TM), AIDSVAX(TM) B/B, AIDSVAX(TM) B/E, HIV gp120 Vaccine - Genentech, HIV gp120 Vaccine AIDSVAX - VaxGen, HIV Vaccine AIDSVAX - VaxGen.* Drugs in R&D, 2003. **4**(4): p. 249-253.
66. Sundling, C., et al., *Soluble HIV-1 Env trimers in adjuvant elicit potent and diverse functional B cell responses in primates.* The Journal of Experimental Medicine, 2010. **207**(9): p. 2003-2017.
67. Sundling, C., et al., *Immunization with Wild-Type or CD4-Binding-Defective HIV-1 Env Trimers Reduces Viremia Equivalently following Heterologous Challenge with Simian-Human Immunodeficiency Virus.* J. Virol., 2010. **84**(18): p. 9086-9095.
68. Nkolola, J.P., et al., *Breadth of Neutralizing Antibodies Elicited by Stable, Homogeneous Clade A and Clade C HIV-1 gp140 Envelope Trimers in Guinea Pigs.* J. Virol., 2010. **84**(7): p. 3270-3279.
69. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand.* New England Journal of Medicine, 2009. **361**(23): p. 2209-2220.
70. Sistigu, A., et al., *Strong CD8+ T cell antigenicity and immunogenicity of large foreign proteins incorporated in HIV-1 VLPs able to induce a Nef-dependent activation/maturation of dendritic cells.* Vaccine, 2011. **29**(18): p. 3465-3475.

71. Ranasinghe, C., et al., *A comparative analysis of HIV-specific mucosal/systemic T cell immunity and avidity following rDNA/rFPV and poxvirus-poxvirus prime boost immunisations.* Vaccine, 2011. **29**(16): p. 3008-3020.
72. Nanjundappa, R.H., et al., *GP120-specific exosome-targeted T cell-based vaccine capable of stimulating DC- and CD4+ T-independent CTL responses.* Vaccine, 2011. **29**(19): p. 3538-3547.
73. Streeck, H. and D.F. Nixon, *T Cell Immunity in Acute HIV-1 Infection.* Journal of Infectious Diseases, 2010. **202**(Supplement 2): p. S302-S308.
74. Wilson, N.A., et al., *Vaccine-Induced Cellular Responses Control Simian Immunodeficiency Virus Replication after Heterologous Challenge.* J. Virol., 2009. **83**(13): p. 6508-6521.
75. Sekaly, R.-P., *The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development?* The Journal of Experimental Medicine, 2008. **205**(1): p. 7-12.
76. Jose Esparza, W.L.H., Saladin Osmanov, *HIV Vaccine Development.* AIDS, 1996. **10**(suppl): p. S123-S132.
77. Kolte, L., et al., *Dysregulation of CD4+CD25+CD127lowFOXP3+ regulatory T cells in HIV-infected pregnant women.* Blood, 2011. **117**(6): p. 1861-1868.
78. Sabado, R.L., et al., *Evidence of dysregulation of dendritic cells in primary HIV infection.* Blood, 2010. **116**(19): p. 3839-3852.
79. Kuhrt, D., et al., *Evidence of Early B-Cell Dysregulation in Simian Immunodeficiency Virus Infection: Rapid Depletion of Naive and Memory B-Cell Subsets with Delayed Reconstitution of the Naive B-Cell Population.* J. Virol., 2010. **84**(5): p. 2466-2476.
80. Fernandez, S., A. Lim, and M. French, *Immune activation and the pathogenesis of HIV disease: implications for therapy.(LEADING ARTICLE)(Report).* Journal of HIV Therapy, 2009. **14**(3): p. 52(5).
81. Chun, T.-W., et al., *Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection.* Nature, 1997. **387**(6629): p. 183-188.
82. Douek, D.C., et al., *HIV preferentially infects HIV-specific CD4+ T cells.* Nature, 2002. **417**(6884): p. 95-98.
83. Finkel, T.H., et al., *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes.* Nat Med, 1995. **1**(2): p. 129-134.
84. D'Orsogna, L.J.a., et al., *Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy.* AIDS, 2007. **21**(13): p. 1747-1752.
85. Fauci, A.S., D. Mavilio, and S. Kottilil, *NK cells in HIV infection: Paradigm for protection or targets for ambush.* Nat Rev Immunol, 2005. **5**(11): p. 835-843.
86. M Roederer, J.G.D., M T Anderson, P A Raju, L A Herzenberg, and L A Herzenberg, *CD8 naive T cell counts decrease progressively in HIV-infected adults.* J Clin Invest., 1995. **95**(5): p. 2061-2066.
87. Michel, P., et al., *Reduced Immune Activation and T Cell Apoptosis in Human Immunodeficiency Virus Type 2 Compared with Type 1: Correlation of T Cell Apoptosis with Œ≤2 Microglobulin Concentration and Disease Evolution.* Journal of Infectious Diseases, 2000. **181**(1): p. 64-75.

88. Baenziger, S., et al., *Triggering TLR7 in mice induces immune activation and lymphoid system disruption, resembling HIV-mediated pathology.* Blood, 2009. **113**(2): p. 377-388.
89. Lapham, C.K., et al., *Evidence for Cell-Surface Association Between Fusin and the CD4-gp120 Complex in Human Cell Lines*, 1996. p. 602-605.
90. Berger, E.A., P.M. Murphy, and J.M. Farber, *CHEMOKINE RECEPTORS AS HIV-1 CORECEPTORS: Roles in Viral Entry, Tropism, and Disease*, 1999. p. 657-700.
91. Fultz, P.N., et al., *Vaccine Protection of Chimpanzees Against Challenge with HIV-1-Infected Peripheral Blood Mononuclear Cells*, 1992. p. 1687-1690.
92. Berman, P.W., et al., *Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160.* Nature, 1990. **345**(6276): p. 622-625.
93. Francis DP, G.T., McElrath MJ, Belshe RB, Gorse GJ, Migasena S, Kitayaporn D, Pitisuttitham P, Matthews T, Schwartz DH, Berman PW, *Advancing AIDSVAX to phase 3. Safety, immunogenicity, and plans for phase 3.* AIDS Res Hum Retroviruses., 1998. **14**(Suppl 3): p. S325-31.
94. Profile, A.R.D., *HIV gp120 Vaccine - VaxGen: AIDSVAX(TM), AIDSVAX(TM) B/B, AIDSVAX(TM) B/E, HIV gp120 Vaccine - Genentech, HIV gp120 Vaccine AIDSVAX - VaxGen, HIV Vaccine AIDSVAX - VaxGen.* Drugs in R&D, 2003. **4**(4): p. 249-253.
95. Polo, J.M. and T.W. Dubensky, *Virus-based vectors for human vaccine applications.* Drug Discovery Today, 2002. **7**(13): p. 719-727.
96. Seaman, M.S., et al., *Multiclade Human Immunodeficiency Virus Type 1 Envelope Immunogens Elicit Broad Cellular and Humoral Immunity in Rhesus Monkeys*, 2005. p. 2956-2963.
97. Mascola, J.R., et al., *Neutralizing Antibodies Elicited by Immunization of Monkeys with DNA Plasmids and Recombinant Adenoviral Vectors Expressing Human Immunodeficiency Virus Type 1 Proteins*, 2005. p. 771-779.
98. Shiver, J.W., et al., *Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity.* Nature, 2002. **415**(6869): p. 331-335.
99. Ferrando-MartÃnez, S., et al., *Differential gag-specific polyfunctional T cell maturation patterns in HIV-1 Elite Controllers*, 2012.
100. Akinsiku, O.T., et al., *Interleukin-2 Production by Polyfunctional HIV-1â€“Specific CD8 T Cells Is Associated With Enhanced Viral Suppression*, 2011. p. 132-140 10.1097/QAI.0b013e318224d2e9.
101. Dharmapuri, S., D. Peruzzi, and L. Aurisicchio, *Engineered adenovirus serotypes for overcoming anti-vector immunity.* Expert Opinion on Biological Therapy, 2009. **9**(10): p. 1279-1287.
102. Lundstrom, K., *Novel developments for applications of alphavirus vectors in gene therapy.* Gene Therapy and Molecular Biology 2001. **6**: p. 25-31.
103. *HIV gp120 Vaccine - VaxGen: AIDSVAX(TM), AIDSVAX(TM) B/B, AIDSVAX(TM) B/E, HIV gp120 Vaccine - Genentech, HIV gp120 Vaccine AIDSVAX - VaxGen, HIV Vaccine AIDSVAX - VaxGen*, 2003. p. 249-253.
104. McBurney, S.P. and T.M. Ross, *Viral sequence diversity: challenges for AIDS vaccine designs.* Expert Review of Vaccines, 2008. **7**(9): p. 1405-1417.
105. Santra, S., et al., *A centralized gene-based HIV-1 vaccine elicits broad cross-clade cellular immune responses in rhesus monkeys.* Proceedings of the National Academy of Sciences, 2008. **105**(30): p. 10489-10494.

106. James Stott , N.A., *Assessing animal models in AIDS.* nature medicine, 1995. **1**(4): p. 295-297.
107. Shedlock, D.J., G. Silvestri, and D.B. Weiner, *Monkeying around with HIV vaccines: using rhesus macaques to define 'gatekeepers' for clinical trials.* Nat Rev Immunol, 2009. **9**(10): p. 717-728.
108. Bendinelli, M., et al., *Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen.* Clin. Microbiol. Rev., 1995. **8**(1): p. 87-112.
109. ELDER, J.H., et al., *Workshop Summary: Lessons from the Cat: Feline Immunodeficiency Virus as a Tool to Develop Intervention Strategies against Human Immunodeficiency Virus Type 1.* AIDS Research and Human Retroviruses, 1998. **14**(9): p. 797-801.
110. Remington, K.M., et al., *Mutants of feline immunodeficiency virus resistant to 3'-azido-3'-deoxythymidine.* J. Virol., 1991. **65**(1): p. 308-312.
111. Burkhard M.Jo, D.G.A., *Transmission and Immunopathogenesis of FIV in Cats as a Model for HIV.* Current HIV Research, 2003. **1**: p. 15-29.
112. Dean, G., et al., *Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node.* J. Virol., 1996. **70**(8): p. 5165-5169.
113. McCune, J.M., *Development and applications of the SCID-hu mouse model.* Seminars in Immunology, 1996. **8**(4): p. 187-196.
114. Goldstein*, H., et al., *SCID-hu mice: a model for studying disseminated HIV infection.* Seminars in Immunology, 1996. **8**(4): p. 223-231.
115. Denton, P.W., et al., *Antiretroviral Pre-exposure Prophylaxis Prevents Vaginal Transmission of HIV-1 in Humanized BLT Mice.* PLoS Med, 2008. **5**(1): p. e16.
116. CULLEN, B.R. and E.D. GARRETT, *A Comparison of Regulatory Features in Primate Lentiviruses.* AIDS Research and Human Retroviruses, 1992. **8**(3): p. 387-393.
117. McClure, H.M., et al., *Nonhuman Primate Models for Evaluation of AIDS Therapya.* Annals of the New York Academy of Sciences, 1990. **616**(1): p. 287-298.
118. Paiardini, M., et al., *Lessons Learned from the Natural Hosts of HIV-Related Viruses.* Annual Review of Medicine, 2009. **60**(1): p. 485-495.
119. Pandrea, I. and C. Apetrei, *Where the Wild Things Are: Pathogenesis of SIV Infection in African Nonhuman Primate Hosts.* Current HIV/AIDS Reports, 2010. **7**(1): p. 28-36.
120. Van Rompay, K.K.A., *Evaluation of antiretrovirals in animal models of HIV infection.* Antiviral Research, 2010. **85**(1): p. 159-175.
121. Ambrose, Z., et al., *HIV/AIDS: in search of an animal model.* Trends in Biotechnology, 2007. **25**(8): p. 333-337.
122. Gardner, M.B. and P.A. Luciw, *Animal models of AIDS,* 1989. p. 2593-2606.
123. McKenna, P., et al., *The Macaque Gut Microbiome in Health, Lentiviral Infection, and Chronic Enterocolitis.* PLoS Pathog, 2008. **4**(2): p. e20.
124. Rhesus Macaque Genome Sequencing and Analysis, C., et al., *Evolutionary and Biomedical Insights from the Rhesus Macaque Genome,* 2007. p. 222-234.
125. Rolland, M., et al., *Reconstruction and Function of Ancestral Center-of-Tree Human Immunodeficiency Virus Type 1 Proteins.* The Journal of Virology, 2007. **81**(16): p. 8507-8514.
126. Nickle, D.C., et al., *Consensus and Ancestral State HIV Vaccines.* Science, 2003. **299**(5612): p. 1515c-1518.

127. Sean, P.M. and M.R. Ted, *Developing Broadly Reactive HIV-1/AIDS Vaccines: A Review of Polyvalent and Centralized HIV-1 Vaccines.* Current Pharmaceutical Design, 2007. **13**: p. 1957-1964.
128. Kothe, D.L., et al., *Ancestral and consensus envelope immunogens for HIV-1 subtype C.* Virology, 2006. **352**(2): p. 438-449.
129. LÃ©tourneau, S., et al., *Design and Pre-Clinical Evaluation of a Universal HIV-1 Vaccine.* PLoS ONE, 2007. **2**(10): p. e984.
130. Yan, J., et al., *Immunogenicity of a novel engineered HIV-1 clade C synthetic consensus-based envelope DNA vaccine.* Vaccine, 2011. **29**(41): p. 7173-7181.
131. Niu, L., et al., *Preclinical evaluation of HIV-1 therapeutic ex vivo dendritic cell vaccines expressing consensus Gag antigens and conserved Gag epitopes.* Vaccine, 2011. **29**(11): p. 2110-2119.
132. Gao, F., et al., *Antigenicity and Immunogenicity of a Synthetic Human Immunodeficiency Virus Type 1 Group M Consensus Envelope Glycoprotein.* The Journal of Virology, 2005. **79**(2): p. 1154-1163.
133. Feng, G., et al., *Centralized HIV-1 Envelope Immunogens and Neutralizing Antibodies.* Current HIV Research, 2007. **5**(6): p. 572-577.
134. Weaver, E.A., et al., *Cross-Subtype T-Cell Immune Responses Induced by a Human Immunodeficiency Virus Type 1 Group M Consensus Env Immunogen.* The Journal of Virology, 2006. **80**(14): p. 6745-6756.
135. Liao, H.-X., et al., *A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses.* Virology, 2006. **353**(2): p. 268-282.
136. Seaman, M.S., et al., *Multiclade Human Immunodeficiency Virus Type 1 Envelope Immunogens Elicit Broad Cellular and Humoral Immunity in Rhesus Monkeys.* The Journal of Virology, 2005. **79**(5): p. 2956-2963.
137. Fischer, W., et al., *Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants.* Nat Med, 2007. **13**(1): p. 100-106.
138. Pal, R., et al., *Definitive toxicology and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 (HIV-1) vaccine in rabbits.* Vaccine, 2006. **24**(8): p. 1225-1234.
139. Wang, S., et al., *Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers.* Vaccine, 2008. **26**(8): p. 1098-1110.
140. Pal, R., et al., *Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate*, 2005. p. 226-236.
141. Mike de Veer, E.M., *New development in vaccine research-unveiling the secret of vaccine adjuvants.* Discov Med, 2011. **12**(64): p. 195-204.
142. Glenny, A.T., *Insoluble precipitates in Diptheria and Tetanus immunization.* Br Med J, 1930. **2**(3632): p. 244-245.
143. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine Adjuvants: Putting Innate Immunity to Work.* Immunity, 2010. **33**(4): p. 492-503.
144. Guy, B., *The perfect mix; recent progress in adjuvant research.* Nat. Rev. Microbiol, 2007. **5**(7): p. 505-517.

145. Pulendran B, A.R., *Immunological mechanisms of vaccination.* Nat. Immunol., 2011. **12**(6): p. 509-17.
146. Bachmann, M.F. and R.M. Zinkernagel, *NEUTRALIZING ANTIVIRAL B CELL RESPONSES.* Annual Review of Immunology, 1997. **15**(1): p. 235-270.
147. Goodridge, H.S., et al., *Activation of the innate immune receptor Dectin-1 upon formation of a /`phagocytic synapse/'.* Nature, 2011. **472**(7344): p. 471-475.
148. Kerrigan, A.M. and G.D. Brown, *Syk-coupled C-type lectins in immunity.* Trends in Immunology, 2011. **32**(4): p. 151-156.
149. Gonzalez, S.F., et al., *Chapter 1 - The Role of Innate Immunity in B Cell Acquisition of Antigen Within LNs*, in *Advances in Immunology*, W.A. Frederick, Editor. 2010, Academic Press. p. 1-19.
150. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nat Rev Immunol, 2010. **10**(11): p. 787-796.
151. Fifis, T., et al., *Size-Dependent Immunogenicity: Therapeutic and Protective Properties of Nano-Vaccines against Tumors.* The Journal of Immunology, 2004. **173**(5): p. 3148-3154.
152. de Veer, M., et al., *The kinetics of soluble and particulate antigen trafficking in the afferent lymph, and its modulation by aluminum-based adjuvant.* Vaccine, 2010. **28**(40): p. 6597-6602.
153. De Veer, M.J., J.M. Kemp, and E.N.T. Meeusen, *The innate host defence against nematode parasites.* Parasite Immunology, 2007. **29**(1): p. 1-9.
154. Kemp, J.M., et al., *Continuous antigen delivery from controlled release implants induces significant and anamnestic immune responses.* Vaccine, 2002. **20**(7,Äì8): p. 1089-1098.
155. Rolf M, Z., *Localization dose and time of antigens determine immune reactivity.* Seminars in Immunology, 2000. **12**(3): p. 163-171.
156. Toapanta, F. and T. Ross, *Complement-mediated activation of the adaptive immune responses.* Immunologic Research, 2006. **36**(1): p. 197-210.
157. Melchers, M., et al., *Targeting HIV-1 Envelope Glycoprotein Trimers to B Cells by Using APRIL Improves Antibody Responses.* Journal of Virology, 2012. **86**(5): p. 2488-2500.
158. Bonifaz, L.C., et al., *In Vivo Targeting of Antigens to Maturing Dendritic Cells via the DEC-205 Receptor Improves T Cell Vaccination.* The Journal of Experimental Medicine, 2004. **199**(6): p. 815-824.
159. Tacken, P.J., et al., *Targeting DC-SIGN via its neck region leads to prolonged antigen residence in early endosomes, delayed lysosomal degradation, and cross-presentation.* Blood, 2011. **118**(15): p. 4111-4119.
160. Harper, D.M., et al., *Sustained efficacy up to 4∑5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial.* The Lancet, 2006. **367**(9518): p. 1247-1255.
161. Allison, A.C. and N.E. Byars, *Immunological adjuvants: Desirable properties and side-effects.* Molecular Immunology, 1991. **28**(3): p. 279-284.
162. James M, B., *(How) do aluminium adjuvants work?* Immunology Letters, 2006. **102**(1): p. 10-15.
163. Morefield, G.L., et al., *Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro.* Vaccine, 2005. **23**(13): p. 1588-1595.
164. Brewer JM, A.J., *Cytokines and the mechanisms of action of vaccine adjuvants.* Cytokines Cell Mol Ther., 1997. **3**(4): p. 233-46.

165. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants.* Current Opinion in Immunology, 2009. **21**(1): p. 23-29.
166. Aucouturier, J., et al., *Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines.* Expert Review of Vaccines, 2002. **1**(1): p. 111-118.
167. Garcon, N., P. Chomez, and M. Van Mechelen, *GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives.* Expert Review of Vaccines, 2007. **6**(5): p. 723-739.
168. Audino, P., *The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine.* Vaccine, 2001. **19**(17,Äì19): p. 2673-2680.
169. Tritto, E., F. Mosca, and E. De Gregorio, *Mechanism of action of licensed vaccine adjuvants.* Vaccine, 2009. **27**(25,Äì26): p. 3331-3334.
170. Aguilar, J.C. and E.G. Rodriguez, *Vaccine adjuvants revisited.* Vaccine, 2007. **25**(19): p. 3752-3762.
171. Ambrosch, F., et al., *Immunogenicity and protectivity of a new liposomal hepatitis A vaccine.* Vaccine, 1997. **15**(11): p. 1209-1213.
172. Ben-Yehuda, A., et al., *Immunogenicity and safety of a novel IL-2-supplemented liposomal influenza vaccine (INFLUSOME-VAC) in nursing-home residents.* Vaccine, 2003. **21**(23): p. 3169-3178.
173. Moser, C., et al., *Influenza virosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations.* Expert Review of Vaccines, 2007. **6**(5): p. 711-721.
174. Herzog, C., et al., *Eleven years of Inflexal¬Æ V,Äîa virosomal adjuvanted influenza vaccine.* Vaccine, 2009. **27**(33): p. 4381-4387.
175. Ruf, B.R., et al., *Open, Randomized Study to Compare the Immunogenicity and Reactogenicity of an Influenza Split Vaccine with an MF59-Adjuvanted Subunit Vaccine and a Virosome-Based Subunit Vaccine in Elderly.* Infection, 2004. **32**(4): p. 191-198.
176. Mike de Veer, M.E., *New developments in vaccine research--unveiling the secret of vaccine adjuvants.* Discov Med, 2011. **12**(64): p. 195-204.
177. Skeiky, Y.A.W., et al., *Differential Immune Responses and Protective Efficacy Induced by Components of a Tuberculosis Polyprotein Vaccine, Mtb72F, Delivered as Naked DNA or Recombinant Protein.* The Journal of Immunology, 2004. **172**(12): p. 7618-7628.
178. Ulrich JT, M.K., *Monophosphoryl lipid A as an adjuvant. Past experiences and new directions.* Pharm Biotechnol., 1995. **6**: p. 495-524.
179. Evans, J.T., et al., *Enhancement of antigen-specific immunity via the TLR4 ligands MPL,Ñ¢ adjuvant and Ribi.529.* Expert Review of Vaccines, 2003. **2**(2): p. 219-229.
180. Higgins, D., et al., *Immunostimulatory DNA as a vaccine adjuvant.* Expert Review of Vaccines, 2007. **6**(5): p. 747-759.
181. Zuber, A.K.m., et al., *Topical delivery of imiquimod to a mouse model as a novel adjuvant for human immunodeficiency virus (HIV) DNA.* Vaccine, 2004. **22**(13,Äì14): p. 1791-1798.
182. Harrison, C.J., R.L. Miller, and D.I. Bernstein, *Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine.* Vaccine, 2001. **19**(13,Äì14): p. 1820-1826.

183. Garcon, N., D.G. Heppner, and J. Cohen, *Development of RTS,S/AS02: a purified subunit-based malaria vaccine candidate formulated with a novel adjuvant.* Expert Review of Vaccines, 2003. **2**(2): p. 231-238.
184. Kensil CR, W.J., Anderson CA, Wheeler DA, Amsden J., *QS-21 and QS-7: purified saponin adjuvants.* Dev Biol Stand., 1998. **92**: p. 41-7.
185. Newman, M.J., et al., *Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations.* Vaccine, 1997. **15**(9): p. 1001-1007.
186. Kim, Y.-J., et al., *Synthetic Studies of Complex Immunostimulants from Quillaja saponaria:,Äâ Synthesis of the Potent Clinical Immunoadjuvant QS-21Aapi.* Journal of the American Chemical Society, 2006. **128**(36): p. 11906-11915.
187. Skene, C.D. and P. Sutton, *Saponin-adjuvanted particulate vaccines for clinical use.* Methods, 2006. **40**(1): p. 53-59.
188. Pearse, M.J. and D. Drane, *ISCOMATRIX¬Æ adjuvant for antigen delivery.* Advanced Drug Delivery Reviews, 2005. **57**(3): p. 465-474.
189. Sun, H.-X., Y. Xie, and Y.-P. Ye, *ISCOMs and ISCOMATRIX,Ñ¢.* Vaccine, 2009. **27**(33): p. 4388-4401.
190. Sanders, M.T., et al., *Single dose intranasal immunization with ISCOMATRIX,Ñ¢ vaccines to elicit antibody-mediated clearance of influenza virus requires delivery to the lower respiratory tract.* Vaccine, 2009. **27**(18): p. 2475-2482.
191. Skene, C.D., C. Doidge, and P. Sutton, *Evaluation of ISCOMATRIX,Ñ¢ and ISCOM,Ñ¢ vaccines for immunisation against Helicobacter pylori.* Vaccine, 2008. **26**(31): p. 3880-3884.
192. Drane, D., et al., *Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX,Ñ¢ vaccine: A phase I study in healthy volunteers.* Human Vaccines & Immunotherapeutics, 2009. **5**(3): p. 151-157.
193. Ebert, L.M., et al., *A Long, Naturally Presented Immunodominant Epitope from NY-ESO-1 Tumor Antigen: Implications for Cancer Vaccine Design.* Cancer Research, 2009. **69**(3): p. 1046-1054.
194. Middleton, D., et al., *Evaluation of Vaccines for H5N1 Influenza Virus in Ferrets Reveals the Potential for Protective Single-Shot Immunization.* Journal of Virology, 2009. **83**(15): p. 7770-7778.
195. Didierlaurent, A.M., et al., *AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity.* The Journal of Immunology, 2009. **183**(10): p. 6186-6197.
196. Stewart, V.A., et al., *Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A.* Vaccine, 2006. **24**(42,Äì43): p. 6483-6492.
197. Eric O Reed, M.A.M., *HIVs and Their Replication*, in *Fields' virology*, P.M.H.a.e. David M. Knipe, Diane E. Griffin ... [et al.], Editor. 2007, Wolters Kluwer Health/Lippincott Williams & Wilkins: Philadelphia. p. 2107-2167.
198. Haynes, B.F. and R.J. Shattock, *Critical issues in mucosal immunity for HIV-1 vaccine development.* The Journal of allergy and clinical immunology, 2008. **122**(1): p. 3-9.
199. Mitchell, J.A., et al., *Induction of heterosubtypic immunity to influenza A virus using a DNA vaccine expressing hemagglutinin-C3d fusion proteins.* Vaccine, 2003. **21**(9-10): p. 902-914.

200. McBurney SP, L.G., Forthal DN, Ross TM, *Evaluation of heterologous vaginal SHIV SF162p4 infection following vaccination with a polyvalent clade B virus-like particle vaccine.* AIDS Research and Human Retroviruses, 2012. **In Press**.
201. Subbarao, S., et al., *Chemoprophylaxis with Tenofovir Disoproxil Fumarate Provided Partial Protection against Infection with Simian Human Immunodeficiency Virus in Macaques Given Multiple Virus Challenges.* The Journal of Infectious Diseases, 2006. **194**(7): p. 904-911.
202. M, E., *Viral quasispecies.* Sci Am, 1993. **269**(1): p. 42-9.
203. Ho, D.D., et al., *Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection.* Nature, 1995. **373**(6510): p. 123-126.
204. Wei, X., et al., *Viral dynamics in human immunodeficiency virus type 1 infection.* Nature, 1995. **373**(6510): p. 117-122.
205. Gao, F., et al., *Antigenicity and Immunogenicity of a Synthetic Human Immunodeficiency Virus Type 1 Group M Consensus Envelope Glycoprotein.* Journal of Virology, 2005. **79**(2): p. 1154-1163.
206. Gaschen B, T.J., Yusim K, Foley B, Gao F, Lang D, Novitsky V, Haynes B, Hahn BH, Bhattacharya T, Korber B., *Diversity considerations in HIV-1 vaccine selection.* Science, 2002. **296**(5577): p. 2354-60.
207. Miguel Arenas, D.P., *Computational Design of Centralized HIV-1 Genes* Current HIV Research, 2010. **8**(8): p. 613-621.
208. Thomson, S.A., et al., *Development of a synthetic consensus sequence scrambled antigen HIV-1 vaccine designed for global use.* Vaccine, 2005. **23**(38): p. 4647-4657.
209. Giles, B.M. and T.M. Ross, *A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets.* Vaccine, 2011. **29**(16): p. 3043-3054.
210. Kesturu, G.S., et al., *Minimization of genetic distances by the consensus, ancestral, and center-of-tree (COT) sequences for HIV-1 variants within an infected individual and the design of reagents to test immune reactivity.* Virology, 2006. **348**(2): p. 437-448.
211. Ross HA, N.D., Liu Y, Heath L, Jensen MA, Rodrigo AG, Mullins JI., *Sources of variation in ancestral sequence reconstruction for HIV-1 envelope genes.* Evol Bioinform Online., 2007. **13**(2): p. 53-76.
212. Zhuang, J., et al., *Human Immunodeficiency Virus Type 1 Recombination: Rate, Fidelity, and Putative Hot Spots.* Journal of Virology, 2002. **76**(22): p. 11273-11282.
213. Jung, A., et al., *Recombination: Multiply infected spleen cells in HIV patients.* Nature, 2002. **418**(6894): p. 144-144.
214. Jost, S., et al., *A Patient with HIV-1 Superinfection.* New England Journal of Medicine, 2002. **347**(10): p. 731-736.
215. Koelsch, K.K., et al., *Clade B HIV-1 superinfection with wild-type virus after primary infection with drug-resistant clade B virus.* AIDS, 2003. **17**(7): p. F11-F16.
216. Posada D, C.K., *The effect of recombination on the accuracy of phylogeny estimation.* J Mol Evol. , 2002. **54**(3): p. 396-402.
217. Schierup, M.H. and J. Hein, *Consequences of Recombination on Traditional Phylogenetic Analysis.* Genetics, 2000. **156**(2): p. 879-891.
218. Pant Pal N, S.S., Cajas JM, *Does Genetic Diversity of HIV-1 Non-B Subtypes Differentially Impact Disease Progression in Treatment-Naive HIV-1 Infected*

Individuals? A Systematic Review of Evidence:1996-2010. J.Acquir. Immune Defic Syndr., 2012. **59**(4): p. 382-8.
219. Gao, F., et al., *Antigenicity and Immunogenicity of a Synthetic Human Immunodeficiency Virus Type 1 Group M Consensus Envelope Glycoprotein*, 2005. p. 1154-1163.
220. Sharma, V.A., et al., *Structural characteristics correlate with immune responses induced by HIV envelope glycoprotein vaccines.* Virology, 2006. **352**(1): p. 131-144.
221. Harris, A., et al., *Trimeric HIV-1 glycoprotein gp140 immunogens and native HIV-1 envelope glycoproteins display the same closed and open quaternary molecular architectures.* PNAS, 2011. **108**(28): p. 11440-11445.
222. Saphire, E.O., et al., *Crystal Structure of a Neutralizing Human IgG Against HIV-1: A Template for Vaccine Design.* Science, 2001. **293**(5532): p. 1155-1159.
223. Parren, P.W.H.I., et al., *Antibody Protects Macaques against Vaginal Challenge with a Pathogenic R5 Simian/Human Immunodeficiency Virus at Serum Levels Giving Complete Neutralization In Vitro*, 2001. p. 8340-8347.
224. Burton, D.R., et al., *Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody*, 2011. p. 11181-11186.
225. Veazey, R.S., et al., *Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120.* Nat Med, 2003. **9**(3): p. 343-346.
226. The rgp, H.I.V.V.S.G., *Placebo-Controlled Phase 3 Trial of a Recombinant Glycoprotein 120 Vaccine to Prevent HIV-1 Infection*, 2005. p. 654-665.
227. Pitisuttithum, P., et al., *Randomized, Double Blind, Placebo Controlled Efficacy Trial of a Bivalent Recombinant Glycoprotein 120 HIV1 Vaccine among Injection Drug Users in Bangkok, Thailand*, 2006. p. 1661-1671.
228. Catanzaro, AndrewÂ T.Â., et al., *Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIVâ€ 1 Candidat Recombinant Adenovirus Vector*, 2006. p. 1638-1649.
229. Russell, N.D., et al., *Phase 2 Study of an HIV-1 Canarypox Vaccine (vCP1452) Alone and in Combination With rgp120: Negative Results Fail to Trigger a Phase 3 Correlates Trial*, 2007. p. 203-212 10.1097/01.qai.0000248356.48501.ff.
230. Catanzaro, A.T., et al., *Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine.* Vaccine, 2007. **25**(20): p. 4085-4092.
231. Graham, Barney S., et al., *Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIV1 DNA Candidate Vaccine*, 2006. p. 1650-1660.
232. Wang, S., et al., *Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA primeâ€“protein boost HIV-1 vaccine in healthy human volunteers.* Vaccine, 2008. **26**(31): p. 3947-3957.
233. Villadangos, J.A. and K. Shortman, *Found in translation: the human equivalent of mouse CD8+ dendritic cells*. p. 1131-1134.
234. Saha R, K.S., Donofrio RS., *DNA vaccines: a mini review*. Recent Pat DNA Gene Seq, 2011. **5**(2): p. 92-6.
235. Vaine, M., S. Lu, and S. Wang, *Progress on the Induction of Neutralizing Antibodies Against HIV Type 1 (HIV-1)*, 2009. p. 137-153 10.2165/00063030-200923030-00001.
236. Deml, L., et al., *Multiple Effects of Codon Usage Optimization on Expression and Immunogenicity of DNA Candidate Vaccines Encoding the Human Immunodeficiency Virus Type 1 Gag Protein*, 2001. p. 10991-11001.

237. Wang, S., et al., *Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine.* Vaccine, 2006. **24**(21): p. 4531-4540.
238. Lu, S., *Combination DNA plus protein HIV vaccines.* Springer Seminars in Immunopathology, 2006. **28**(3): p. 255-265.
239. Beddows, S., et al., *Evaluating the Immunogenicity of a Disulfide-Stabilized, Cleaved, Trimeric Form of the Envelope Glycoprotein Complex of Human Immunodeficiency Virus Type 1*, 2005. p. 8812-8827.
240. Barnett, S.W.a., et al., *Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope.* AIDS, 2008. **22**(3): p. 339-348.
241. Lakhashe, S.K., et al., *Vaccination against Heterologous R5 Clade C SHIV: Prevention of Infection and Correlates of Protection.* PLoS ONE, 2011. **6**(7): p. e22010.
242. Barnett, S.W., et al., *Antibody-Mediated Protection against Mucosal Simian-Human Immunodeficiency Virus Challenge of Macaques Immunized with Alphavirus Replicon Particles and Boosted with Trimeric Envelope Glycoprotein in MF59 Adjuvant*, 2010. p. 5975-5985.
243. Ishizaka, S.T., et al., *IgG Subtype Is Correlated with Efficiency of Passive Protection and Effector Function of Anti-Herpes Simplex Virus Glycoprotein D Monoclonal Antibodies*, 1995. p. 1108-1111.
244. Hessell, A.J., et al., *Broadly Neutralizing Monoclonal Antibodies 2F5 and 4E10 Directed against the Human Immunodeficiency Virus Type 1 gp41 Membrane-Proximal External Region Protect against Mucosal Challenge by Simian-Human Immunodeficiency Virus SHIVBa-L*, 2009. p. 1302-1313.
245. Watkins, J.D., et al., *An Anti-HIV-1 V3 Loop Antibody Fully Protects Cross-Clade and Elicits T-Cell Immunity in Macaques Mucosally Challenged with an R5 Clade C SHIV.* PLoS ONE, 2011. **6**(3): p. e18207.
246. Palombi, L., et al., *Immunologic Response to Highly Active Antiretroviral Therapy and Mortality Reduction in a Cohort of Human Immunodeficiency Virus Positive Persons in Mozambique.* Am J Trop Med Hyg, 2010. **83**(5): p. 1128-1132.
247. Girard, M.P., et al., *Human immunodeficiency virus (HIV) immunopathogenesis and vaccine development: A review.* Vaccine. **29**(37): p. 6191-6218.
248. Joris, H., *The origin and diversity of the HIV-1 pandemic.* Trends in Molecular Medicine, 2012(0).
249. Gaschen, B., et al., *Diversity Considerations in HIV-1 Vaccine Selection.* Science, 2002. **296**(5577): p. 2354-2360.
250. Spira, S., et al., *Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance.* Journal of Antimicrobial Chemotherapy, 2003. **51**(2): p. 229-240.
251. Joris, H., *The origin and diversity of the HIV-1 pandemic.* Trends in Molecular Medicine, (0).
252. Lu, S., J. M. Grimes Serrano, and S. Wang, *Polyvalent AIDS Vaccines.* Current HIV Research, 2010. **8**(8): p. 622-629.
253. Arenas, M. and D. Posada, *Computational Design of Centralized HIV-1 Genes.* Current HIV Research, 2010. **8**(8): p. 613-621.

254. Gao, F., et al., *Centralized HIV-1 Envelope Immunogens and Neutralizing Antibodies.* Current HIV Research, 2007. **5**(6): p. 572-577.
255. Wang, S., et al., *Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA primeâ€“protein boost HIV-1 vaccine in healthy human volunteers.* Vaccine, 2008. **26**(8): p. 1098-1110.
256. Yan, J., et al., *Enhanced Cellular Immune Responses Elicited by an Engineered HIV-1 Subtype B Consensus-based Envelope DNA Vaccine.* Mol Ther, 2007. **15**(2): p. 411-421.
257. McBurney, S.P. and T.M. Ross, *Human immunodeficiency virus-like particles with consensus envelopes elicited broader cell-mediated peripheral and mucosal immune responses than polyvalent and monovalent Env vaccines.* Vaccine, 2009. **27**(32): p. 4337-4349.
258. Santra, S., et al., *A centralized gene-based HIV-1 vaccine elicits broad cross-clade cellular immune responses in rhesus monkeys.* PNAS, 2008. **105**(30): p. 10489-10494.
259. Barnett, S.W., et al., *Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope.* AIDS, 2008. **22**(3): p. 339-348 10.1097/QAD.0b013e3282f3ca57.
260. Barnett, S.W., et al., *Antibody-Mediated Protection against Mucosal Simian-Human Immunodeficiency Virus Challenge of Macaques Immunized with Alphavirus Replicon Particles and Boosted with Trimeric Envelope Glycoprotein in MF59 Adjuvant.* Journal of Virology, 2010. **84**(12): p. 5975-5985.
261. Kothe, D.L., et al., *Antigenicity and immunogenicity of HIV-1 consensus subtype B envelope glycoproteins.* Virology, 2007. **360**(1): p. 218-234.
262. Barouch, D.H., et al., *Vaccine protection against acquisition of neutralization-resistant SIV challenges in rhesus monkeys.* Nature, 2012. **advance online publication**.
263. Holl, V., M. Peressin, and C. Moog, *Antibody-Mediated Fcψ Receptor-Based Mechanisms of HIV Inhibition: Recent Findings and New Vaccination Strategies.* Viruses, 2009. **1**(3): p. 1265-1294.
264. Burton, D.R., *Antibodies, viruses and vaccines.* Nat Rev Immunol, 2002. **2**(9): p. 706-713.
265. Haynes, B. *Case control study of the RV144 trial for immune correlates: the analysis and way forward*. in *AIDS Vaccine Conference 2011*. 2011. Bangkok, Thailand.
266. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand.* The New England Journal of Medicine, 2009. **361**(23): p. 2209-2220.
267. Loffredo, J.T., et al., *Mamu-B*08-Positive Macaques Control Simian Immunodeficiency Virus Replication.* Journal of Virology, 2007. **81**(16): p. 8827-8832.
268. Smith, M., F. Wightman, and S. Lewin, *HIV Reservoirs and Strategies for Eradication.* Current HIV/AIDS Reports, 2012: p. 1-11.
269. Veazey, R.S., et al., *Increased Loss of CCR5+ CD45RAâˆ’ CD4+ T Cells in CD8+ Lymphocyte-Depleted Simian Immunodeficiency Virus-Infected Rhesus Monkeys*, 2008. p. 5618-5630.
270. Hem, S.L., C.T. Johnston, and H. HogenEsch, *Imject Alum is not aluminum hydroxide adjuvant or aluminum phosphate adjuvant.* Vaccine, 2007. **25**(27): p. 4985-4986.
271. Didierlaurent, A.M., et al., *AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity.* The Journal of Immunology, 2009. **183**(10): p. 6186-6197.

272. Giannini, S.L., et al., *Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only.* Vaccine, 2006. **24**(33â€“34): p. 5937-5949.
273. Sundling, C., et al., *Soluble HIV-1 Env trimers in adjuvant elicit potent and diverse functional B cell responses in primates.* The Journal of Experimental Medicine, 2010. **207**(9): p. 2003-2017.
274. Leroux-Roels, I., et al., *Strong and persistent CD4+ T-cell response in healthy adults immunized with a candidate HIV-1 vaccine containing gp120, Nef and Tat antigens formulated in three Adjuvant Systems.* Vaccine, 2010. **28**(43): p. 7016-7024.
275. Gavioli, R., et al., *The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: Implications for the design of new vaccination strategies against AIDS.* Vaccine, 2008. **26**(5): p. 727-737.
276. Ferrantelli, F., et al., *A combination HIV vaccine based on Tat and Env proteins was immunogenic and protected macaques from mucosal SHIV challenge in a pilot study.* Vaccine, 2011. **29**(16): p. 2918-2932.
277. Ferre, A.L., et al., *Immunodominant HIV-Specific CD8+ T-Cell Responses Are Common to Blood and Gastrointestinal Mucosa, and Gag-Specific Responses Dominate in Rectal Mucosa of HIV Controllers.* JVI, 2010. **84**(19): p. 10354-10365.
278. Ranasinghe, S., et al., *HIV-Specific CD4 T Cell Responses to Different Viral Proteins Have Discordant Associations with Viral Load and Clinical Outcome.* JVI, 2011. **86**(1): p. 277-283.
279. UNAIDS, *Global HIV/AIDS Response: Epidemic update and health sector progress towards Universal Acess- Progress Report 2011*, 2011.
280. Hall, H.I., et al., *Estimation of HIV Incidence in the United States.* JAMA: The Journal of the American Medical Association, 2008. **300**(5): p. 520-529.
281. (CDC), C.f.D.C.a.P., *Persons tested for HIV--United States, 2006.*, in *MMWR Morb Mortal Wkly Rep.*2008, CDC. p. 845-9.
282. *District of Columbia Department of Health Report on HIV/AIDS, 2008*, 2008.
283. Hinman, A., *ERADICATION OF VACCINE-PREVENTABLE DISEASES.* Annual Review of Public Health, 1999. **20**(1): p. 211-229.
284. Mascola, J.R. and D.C. Montefiori, *The Role of Antibodies in HIV Vaccines.* Annual Review of Immunology, 2010. **28**(1): p. 413-444.
285. Ross, A.L., et al., *Progress towards development of an HIV vaccine: report of the AIDS Vaccine 2009 Conference.* The Lancet Infectious Diseases, 2010. **10**(5): p. 305-316.
286. McElrath, M.J. and B.F. Haynes, *Induction of Immunity to Human Immunodeficiency Virus Type-1 by Vaccination.* Immunity, 2010. **33**(4): p. 542-554.
287. Haase, A.T., *Targeting early infection to prevent HIV-1 mucosal transmission.* Nature, 2010. **464**(7286): p. 217-223.
288. McMichael, A.J., et al., *The immune response during acute HIV-1 infection: clues for vaccine development.* Nat Rev Immunol, 2010. **10**(1): p. 11-23.
289. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand*, 2009. p. 2209-2220.
290. Koup RA, P.C., Mazzara G, Panicali D, Sullivan JL., *Broadly reactive antibody-dependent cellular cytotoxic response to HIV-1 envelope glycoproteins precedes broad neutralizing response in human infection.* Viral Immunol, 1991. **4**(4): p. 215-23.

291. David C. Montefiori, a.L.M., b Guido Ferrari,a and John R. Mascola, *Neutralizing and other antiviral antibodies in HIV-1 infection and vaccination.* Curr Opin HIV AIDS, 2007. **2**(3): p. 169-176.
292. Holl, V., et al., *Efficient inhibition of HIV-1 replication in human immature monocyte-derived dendritic cells by purified anti¬ñHIV-1 IgG without induction of maturation.* Blood, 2006. **107**(11): p. 4466-4474.
293. Holl, V., et al., *Nonneutralizing Antibodies Are Able To Inhibit Human Immunodeficiency Virus Type 1 Replication in Macrophages and Immature Dendritic Cells.* Journal of Virology, 2006. **80**(12): p. 6177-6181.
294. FE., M., *Understanding the genetic diversity of HIV-1.* AIDS, 2000. **14**(Suppl 3): p. S31-44.
295. Wyatt R, S.J., *The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.* Science, 1998. **280**(5371): p. 1884-8.
296. Pantophlet, R. and D.R. Burton, *GP120: Target for Neutralizing HIV-1 Antibodies.* Annual Review of Immunology, 2006. **24**(1): p. 739-769.
297. Kwong, P.D., et al., *HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites.* Nature, 2002. **420**(6916): p. 678-682.
298. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-312.
299. Liao HX, S.L., Xia SM, Brock ME, Scearce RM, Vanleeuwen S, Alam SM, McAdams M, Weaver EA, Camacho Z, Ma BJ, Li Y, Decker JM, Nabel GJ, Montefiori DC, Hahn BH, Korber BT, Gao F, Haynes BF., *A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses.* Virology, 2006. **353**(2): p. 268.
300. McBurney SP, L.G., Forthal DN, Ross TM., *Evaluation of heterologous vaginal SHIV SF162p4 infection following vaccination with a polyvalent clade B virus-like particle vaccine.* AIDS Res Hum Retroviruses., 2012.
301. Binley, J.M., et al., *Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies.* Journal of Virology, 2004. **78**(23): p. 13232-13252.
302. Ringe, R., S. Phogat, and J. Bhattacharya, *Subtle alteration of residues including N-linked glycans in V2 loop modulate HIV-1 neutralization by PG9 and PG16 monoclonal antibodies.* Virology, 2012. **426**(1): p. 34-41.
303. Krachmarov, C., et al., *Characterization of Structural Features and Diversity of Variable-Region Determinants of Related Quaternary Epitopes Recognized by Human and Rhesus Macaque Monoclonal Antibodies Possessing Unusually Potent Neutralizing Activities.* Journal of Virology, 2011. **85**(20): p. 10730-10740.
304. Dey, B., C.S. Del Castillo, and E.A. Berger, *Neutralization of Human Immunodeficiency Virus Type 1 by sCD4-17b, a Single-Chain Chimeric Protein, Based on Sequential Interaction of gp120 with CD4 and Coreceptor.* Journal of Virology, 2003. **77**(5): p. 2859-2865.
305. Wang, S., et al., *Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime,Äìprotein boost HIV-1 vaccine in healthy human volunteers.* Vaccine, 2008. **26**(8): p. 1098-1110.

306. Lovelace, E., et al., *The role of amino acid changes in the human immunodeficiency virus type 1 transmembrane domain in antibody binding and neutralization.* Virology, 2011. **421**(2): p. 235-244.
307. McLellan, J.S., et al., *Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9.* Nature, 2011. **480**(7377): p. 336-343.
308. Walker, L.M., et al., *Broad neutralization coverage of HIV by multiple highly potent antibodies.* Nature, 2011. **477**(7365): p. 466-470.
309. Walker, L.M., et al., *Broad and Potent Neutralizing Antibodies from an African Donor Reveal a New HIV-1 Vaccine Target.* Science, 2009. **326**(5950): p. 285-289.
310. JR., M., *Defining the protective antibody response for HIV-1.* Curr Mol Med, 2003. **3**: p. 211-218.
311. Dickover, R., et al., *Role of Maternal Autologous Neutralizing Antibody in Selective Perinatal Transmission of Human Immunodeficiency Virus Type 1 Escape Variants.* Journal of Virology, 2006. **80**(13): p. 6525-6533.
312. Wu, X., et al., *Neutralization Escape Variants of Human Immunodeficiency Virus Type 1 Are Transmitted from Mother to Infant.* Journal of Virology, 2006. **80**(2): p. 835-844.
313. Smith DM, S.M., Frost SD, Pillai SK, Wong JK, Wrin T, Liu Y, Petropolous CJ, Daar ES, Little SJ, Richman DD., *Lack of neutralizing antibody response to HIV-1 predisposes to superinfection.* Virology., 2006. **355**(1): p. 1-5.
314. Yuste, E., et al., *Simian Immunodeficiency Virus Engrafted with Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Epitopes: Replication, Neutralization, and Survey of HIV-1-Positive Plasma.* Journal of Virology, 2006. **80**(6): p. 3030-3041.
315. Binley, J.M., et al., *Profiling the Specificity of Neutralizing Antibodies in a Large Panel of Plasmas from Patients Chronically Infected with Human Immunodeficiency Virus Type 1 Subtypes B and C.* Journal of Virology, 2008. **82**(23): p. 11651-11668.
316. Alam, S.M., et al., *The Role of Antibody Polyspecificity and Lipid Reactivity in Binding of Broadly Neutralizing Anti-HIV-1 Envelope Human Monoclonal Antibodies 2F5 and 4E10 to Glycoprotein 41 Membrane Proximal Envelope Epitopes.* The Journal of Immunology, 2007. **178**(7): p. 4424-4435.
317. Saphire EO, P.P., Pantophlet R, Zwick MB, Morris GM, Rudd PM, Dwek RA, Stanfield RL, Burton DR, Wilson IA., *Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design.* Science, 2001. **293**(5532): p. 1155-9.
318. Montefiori, D.C. and J.R. Mascola, *Neutralizing antibodies against HIV-1: can we elicit them with vaccines and how much do we need?* Current Opinion in HIV and AIDS, 2009. **4**(5): p. 347-351 10.1097/COH.0b013e32832f4a4d.
319. Hem, S.L., C.T. Johnston, and H. HogenEsch, *Imject Alum is not aluminum hydroxide adjuvant or aluminum phosphate adjuvant.* Vaccine, 2007. **25**(27): p. 4985-4986.
320. Martinon, F., A. Mayor, and J.r. Tschopp, *The Inflammasomes: Guardians of the Body.* Annual Review of Immunology, 2009. **27**(1): p. 229-265.
321. Li, H., S. Nookala, and F. Re, *Aluminum Hydroxide Adjuvants Activate Caspase-1 and Induce IL-1Œ≤ and IL-18 Release.* The Journal of Immunology, 2007. **178**(8): p. 5271-5276.
322. Dey, A.K. and I.K. Srivastava, *Novel adjuvants and delivery systems for enhancing immune responses induced by immunogens.* Expert Review of Vaccines, 2010. **10**(2): p. 227-251.

323. Daniel, M.D., et al., *Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene*, 1992. p. 1938-1941.
324. Berry, N., et al., *Early Potent Protection against Heterologous SIVsmE660 Challenge Following Live Attenuated SIV Vaccination in Mauritian Cynomolgus Macaques.* PLoS ONE, 2011. **6**(8): p. e23092.
325. Johnson, R.P., et al., *Highly Attenuated Vaccine Strains of Simian Immunodeficiency Virus Protect against Vaginal Challenge: Inverse Relationship of Degree of Protection with Level of Attenuation*, 1999. p. 4952-4961.
326. Miller, A., et al., *Sindbis virus vectors elicit hemagglutinin-specific humoral and cellular immune responses and offer a dose-sparing strategy for vaccination.* Vaccine, 2008. **26**(44): p. 5641-5648.
327. Ljungberg, K., et al., *Increased Immunogenicity of a DNA-Launched Venezuelan Equine Encephalitis Virus-Based Replicon DNA Vaccine.* Journal of Virology, 2007. **81**(24): p. 13412-13423.
328. Leitner, W.W., et al., *Enhancement of Tumor-specific Immune Response with Plasmid DNA Replicon Vectors*, 2000. p. 51-55.
329. Rayner, J.O., S.A. Dryga, and K.I. Kamrud, *Alphavirus vectors and vaccination.* Reviews in Medical Virology, 2002. **12**(5): p. 279-296.
330. Wong, J.K., et al., *In Vivo CD8+ T-Cell Suppression of SIV Viremia Is Not Mediated by CTL Clearance of Productively Infected Cells.* PLoS Pathog, 2008. **6**(1): p. e1000748.
331. Malkevitch, N.V., et al., *Durable protection of rhesus macaques immunized with a replicating adenovirus-SIV multigene prime/protein boost vaccine regimen against a second SIVmac251 rectal challenge: Role of SIV-specific CD8+ T cell responses.* Virology, 2006. **353**(1): p. 83-98.
332. Hessell, A.J., et al., *Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques.* Nat Med, 2009. **15**(8): p. 951-954.
333. Hessell, A.J., et al., *Broadly Neutralizing Human Anti-HIV Antibody 2G12 Is Effective in Protection against Mucosal SHIV Challenge Even at Low Serum Neutralizing Titers.* PLoS Pathog, 2009. **5**(5): p. e1000433.
334. Hessell, A.J., et al., *Fc receptor but not complement binding is important in antibody protection against HIV.* Nature, 2007. **449**(7158): p. 101-104.
335. Morris, J.O.a.L., *The Antibody Response against HIV-1.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(1).
336. Allen, C.D.C., T. Okada, and J.G. Cyster, *Germinal-Center Organization and Cellular Dynamics.* Immunity, 2007. **27**(2): p. 190-202.
337. MacLennan, I.C.M., *Germinal Centers.* Annual Review of Immunology, 1994. **12**(1): p. 117-139.
338. Lakhashe, S.K., et al., *Vaccination against Heterologous R5 Clade C SHIV: Prevention of Infection and Correlates of Protection.* PLoS ONE, 2011. **6**(7): p. e22010.
339. Lakhashe, S.K., et al., *Prime boost vaccination with heterologous live vectors encoding SIV gag and multimeric HIV-1 gp160 protein: Efficacy against repeated mucosal R5 clade C SHIV challenges.* Vaccine, 2011. **29**(34): p. 5611-5622.
340. Cox, J.H., et al., *Inclusion of a CRF01_AE HIV envelope protein boost with a DNA/MVA prime-boost vaccine: Impact on humoral and cellular immunogenicity and viral load reduction after SHIV-E challenge.* Vaccine, 2012. **30**(10): p. 1830-1840.