

**CONSTRUCTION, CHARACTERIZATION AND IMMUNOGENICITY OF
HUMAN IMMUNODEFICIENCY VIRUS-LIKE PARTICLES**

by

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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

2005

UNIVERSITY OF PITTSBURGH

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A vaccine expressing virus-like particles is an attractive candidate for the development of an effective vaccine for human immunodeficiency virus type 1 (HIV-1). A single vaccine plasmid was constructed to express HIV-1 Gag, Pol, Env, Tat, Rev and Vpu. Safety mutations and deletions were introduced into the VLP DNA to generate a vaccine insert that was non-infectious. The 5' and 3' long terminal repeats, integrase, *vif*, *vpr* and *nef* were removed to further enhance the safety of the vaccine insert. Moreover, mutations were introduced into nucleocapsid and reverse transcriptase to severely restrict viral RNA packaging and to abolish RT and RNase H activity. Virus-like particles were efficiently released from primate cells, but particles were not produced in rodent cells. Therefore, purified particles were used as the inoculum to test the immunogenicity of the VLP vaccines in a rodent system. Systemic and mucosal immune responses to HIV-1 were enhanced by intranasal immunization of purified VLPs expressed from the virally-regulated multi-gene DNA vaccine. VLPs were co-immunized with cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs) to enhance the immune response to HIV-1 gene products. VLPs elicited specific immunity to HIV-1 antigens in both the systemic and mucosal immune compartments. Anti-Env antibodies were detected in the sera, as well as in the washes from harvested lungs, intestines and vagina from immunized mice. In addition, Env- and Gag-specific IFN- γ -secreting splenocytes were elicited in the mice vaccinated with VLPs. Co-inoculation of CpG ODNs with VLPs significantly enhanced both arms of the immune response. In addition, these particulate immunogens were compared to soluble proteins (Gag and Env). Mice immunized with soluble protein alone or co-vaccinated with CpG ODNs elicited much lower immune responses compared to VLP-vaccinated mice. Specifically, CTLs were induced by vaccination with VLPs, whereas the immune response elicited by soluble proteins (+/- CpG ODNs) was almost exclusively antibody-mediated. Overall, a vaccine expressing virus-like particles is one of the most promising alternatives to replication-competent virus in eliciting high levels of cross-reactive neutralizing antibodies in combination with a robust cell-mediated response against multiple viral antigens to protect an HIV-infected host from life-long infection or disease.

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ABBREVIATIONS

ADCC = antibody dependent cellular cytotoxicity
AIDS = acquired immune deficiency syndrome
Amp = ampicillin
APC = antigen presenting cell
ARRRP = AIDS Reference and Reagent Program
ARV = AIDS-associated retrovirus
ATCC = America Type Culture Collection
 β -gal = beta galactosidase
BGH poly A = bovine growth hormone polyadenylation signal
BSA = bovine serum albumin
CA = capsid
CAEV = caprine arthritis encephalitis virus
CCD = charged-couple device
CCR = chemokine coreceptor
cDMEM = complete Dulbecco's modified eagle medium
CMV-IE = cytomegalovirus immediate early
CpG ODN = cytosine phosphate guanosine oligodeoxynucleotides
CRF = recombinant circulating forms
CT = cholera toxin
CTE = constitutive transport element
CTL = cytotoxic T lymphocyte
CTS = central termination signal
CypA = cyclophilin A
ddNTP = dideoxynucleotide triphosphate
dNTP = dideoxynucleotide triphosphate
DNA = deoxyribonucleic acid
dsDNA = double-stranded DNA
EIAV = equine infectious anemia virus
ELISA = enzyme linked immunosorbent assay

ELISPOT = enzyme linked immuno-spot
EM = electron microscopy
EMCV = encephalomyocarditis virus
Env = envelope
Env_{fl} = full length Env
Env_t = truncated Env
FIV = feline immunodeficiency virus
FPV = fowlpox virus
Gag = group associated antigen
g.g. = gene gun
HAART = highly active antiretroviral treatment
HIV = human immunodeficiency virus
HRP = horseradish peroxidase
HTLV = human T-cell lymphotropic virus type
I = interacting domain
i.d. = intradermal
Ig = immunoglobulin
IL = interleukin
i.m. = intramuscular
INF = interferon
IN = integrase
i.p. = intraperitoneal
i.v. = intravenous
Kan = kanamycin
L = late domain
LAV = lymphadenopathy-associated virus
LDH = lactate dehydrogenase
LT = labile toxin
LTNP = long-term non-progressor
LTR = long terminal repeat
LTS = long-term survivors

LUC = luciferase
M = membrane-binding domain
M = main group
MA = matrix
MCS = multiple cloning site
MHC = major histocompatibility complex
MHR = major homology region
MID = monkey infectious dose
MPMV = Mason-Pfizer monkey virus
mRNA = messenger RNA
M-tropic = macrophage-tropic
MuLV = murine leukemia virus
MVA = modified vaccinia Ankara
MVB = microvesicular body
N = non group M/O
NALT = nasal-associated lymphoid tissue
NC = nucleocapsid
Nef = negative regulatory factor
NES = nuclear export signal
NIH = National Institute of Health
NLS = nuclear localization signal
NRE = negative regulatory element
O = outlier group
O.D. = optical density
PAGE = polyacrylamide gel electrophoresis
PBMC = peripheral blood mononuclear cells
pbs = primer binding site
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PIC = preintegration complex
PNP = paranitrophenol

Pol = polymerase
PPT = polypurine tract
PR = protease
pr55 = Gag-Pol precursor polyprotein
p.v. = post vaccination
rBV = recombinant baculovirus
Rev = regulator of virion protein expression
RLU = relative light units
RNA = ribonucleic acid
RRE = rev response element
RT = reverse transcriptase
rVV = recombinant vaccinia virus
SBBC = Sydney Blood Bank Cohort
SD = standard deviation
SDM PCR = site-directed mutagenesis PCR
SDS = sodium dodecyl sulfate
SEAP = secreted alkaline phosphatase
SHIV = simian-human immunodeficiency virus
SIV = simian immunodeficiency virus
ssRNA = single-stranded RNA
SU = surface
SV40 = simian virus 40
TAK = Tat-associated kinase
TAR = *trans*-activation response
Tat = trans-activator of transcription
TCA = trichloroacetic acid
TLR = Toll-like receptor
TM = transmembrane
tRNA = transfer RNA
T-tropic = T cell tropic
USDA = United States Department of Agriculture

VERT = Vpu, Env, Rev, Tat

Vif = virion infectivity factor

VLP = virus-like particle

Vpr = viral protein r

Vpu = viral protein u

Vpx = viral protein x

vRNA = viral RNA

WHO = World Health Organization

ZDV = zidovudine

PREFACE

There are numerous people I would like to thank for their contribution to my graduate career. I would like to first thank my advisor, Dr. Ted Ross, for all of his support and guidance throughout the past five years. I would also like to thank both “sets” of my thesis committee for their insight and assistance in my graduate education: Dr. Richard Franklin, Dr. Thomas McConnell, Dr. James McCubrey, Dr. Stephanie Oberhaus (East Carolina University), Dr. Velpandi Ayyavoo, Dr. Sharon Hillier, Dr. Ronald Montelaro and Dr. Kelly Stefano-Cole (University of Pittsburgh). Also, there have been many members in the Ross laboratory. I’d like to thank Tom Green for my initial training. I would also like to extend a special thanks to Dr. Joseph Bower and Dr. Franklin Toapanta for all of their technical assistance and intellectual contribution to my project and sanity. Without my lab “brothers”, I would still be harvesting spleens...

I would also like to thank my family. Jennifer, Lori, Joey and Butchie-I would like to thank you for always reminding me that there is a life outside the lab but allowing me to be the family nerd just the same. Lastly, I would like to thank my parents, Joseph and Anna Marie Young. I know I never would have completed my Ph.D. without the two of you behind me. However, I also know that no matter what I chose to do, Pizza Hut or Ph.D., you would have been there, just like you have been there my entire life, and for that I am eternally grateful. No words can come close to expressing my appreciation for everything you have sacrificed for me.

I. Chapter 1: Introduction

This chapter was modified with permission from:

Young, KR and TM Ross.

Particle-Based Vaccines for HIV-1 Infection.

***Current Drug Targets-Infectious Disorders*, 2003, 3, pp. 151-169.**

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I.A. Global Impact of the AIDS Epidemic

At the end of 2004, approximately 39.4 million people were infected worldwide with human immunodeficiency virus type 1 (HIV-1), the causative agent of Acquired Immune Deficiency Syndrome (AIDS). Greater than 95% of HIV infections occur in developing countries (25.4 million infected people living in Sub-Saharan Africa and 8.2 million infected individuals reside in Asia)²⁸⁶. It is estimated that one-fourth of the one million United States (U.S.) residents living with HIV are unaware that they are infected⁴⁷⁴. In addition, half of the 400,000 newly infected individuals are younger than 25 years of age each year in the U.S. (70% men, 30% women)⁹¹. HIV/AIDS will continue to threaten human life and health, especially in developing countries, until an effective vaccine is developed.

I.B. Taxonomy

Viruses in the retrovirus family, *Retroviridae*, generally contain an RNA genome that is converted to a DNA form and integrates into the host chromosomal DNA. There are simple (alpharetroviruses, betaretroviruses, gammaretroviruses) and complex (deltaretroviruses, epsilonretroviruses, lentiviruses, spumaviruses) retroviruses. The simple viruses only contain the *gag*, RT, PR and *env* genes, whereas the complex retroviruses contain these 4 genes plus an assortment of regulatory and accessory genes.

HIV-1 is a complex virus member of the *Lentivirus* genus along with HIV type 2 (HIV-2), simian immunodeficiency virus (SIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), caprine arthritis encephalitis virus (CAEV) and visna/maedi virus. Lentiviruses are characteristically identified by cylindrical or cone-like cores. The genome of all lentiviruses contains the *gag*, *PR*, *RT* and *env* genes along with small regulatory proteins that assist in transcription, RNA processing, virion assembly, host gene expression and many other functions in the replication cycle¹⁶¹.

HIV-1 was isolated and determined to be the causative agent of AIDS in 1983^{31, 520}. As a result, each laboratory gave each isolate a different name for this virus: lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), and AIDS-associated retrovirus (ARV). In 1986, the International Committee on Taxonomy of Viruses named this virus, HIV-1.

HIV-1, HIV-2 and SIV are examples of primate lentiviruses of the *Retroviridae* family^{121, 530, 668}. Each virus is able to induce an acquired immunodeficiency disease in the respective host^{198, 488}. HIV-1 is genotypically divided into three distinct groups: major (M), outlier (O), and non-M/non-O (N). The strains of HIV-1 that infect most humans worldwide are found within the M group. Since its introduction into the human population, the M group has evolved into at least 10 distinct clades (A, B, C, D, F, G, H and J) and 13 different circulating recombinant forms (CRF)^{394, 470}. Interestingly, HIV-2 is more closely related to virus strains isolated from sooty mangabey monkeys, *SIV_{sm}*, than HIV-1²⁶⁷. The genetic diversity of HIV has been studied using group M viruses of different geographic origins^{393, 394, 622}. The genetic diversity of the HIV-1 population can vary from 6-10% within an infected individual. Moreover, intraclade

nucleotide diversity can vary 15% (Gag) or up to 30% (Env_{gp120}), whereas interclade variability may range between 30-40% depending on the gene examined.

I.C. HIV-1 Virus and Genome

HIV-1 is an enveloped RNA virus (100-120 nm) (Figure 1). The envelope is composed of a lipid bilayer derived from the host cell membrane during the budding process and is embedded with multimeric glycoproteins. Each envelope (Env; 7-15 Env trimers per virion) glycoprotein, is composed of a surface, globular domain (gp120, SU) and a transmembrane domain (gp41, TM). The matrix (MA, p17) protein lines the inner surface of the viral envelope and surrounds the capsid. The capsid (CA, p24) layer contains approximately 2,000 molecules and encases the nucleocapsid (NC, p9), which surrounds the viral genome and associated viral proteins.

The HIV-1 proviral genome consists of double-stranded DNA (dsDNA) that contains 9 open reading frames that encode for 15 viral proteins⁶⁴² (Figure 1 and Table 1). Similar to all retroviruses, HIV contains three major genes (*gag*, *pol* and *env*), which encode polyprotein precursors that are cleaved to yield the core structural (CA, MA, NC), enzymatic (PR, RT, IN) and envelope (gp120, gp41) proteins, respectively. The HIV-1 genome contains two regulatory genes (*tat* and *rev*) and 4 accessory genes (*nef*, *vif*, *vpu*, and *vpr*) that are required for efficient virion replication and maturation (Table 1). There are two long terminal repeat (LTRs) which flank both the 5' and 3' ends of the proviral DNA genome. The 5' LTR contains the HIV-1 promoter and enhancer sequences that regulate gene expression.

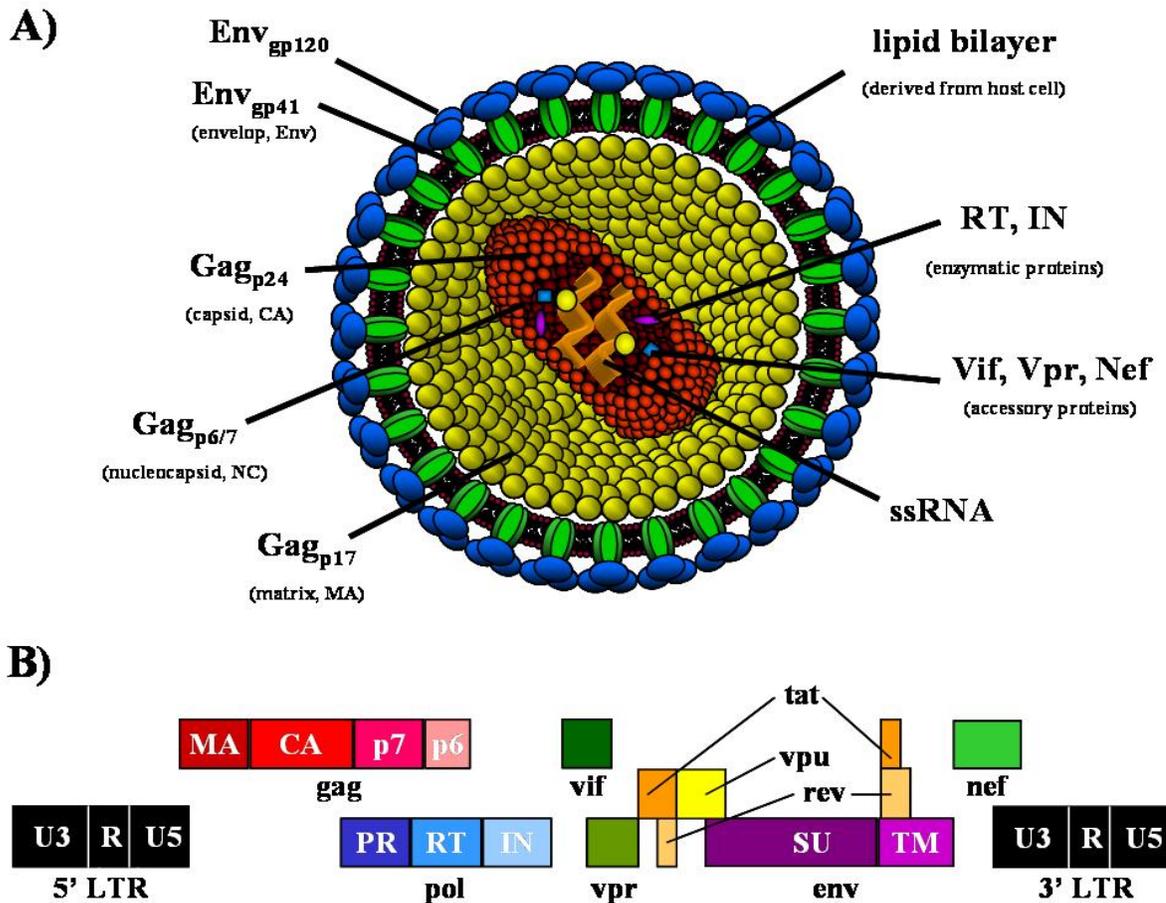


Figure 1. Schematic representation of HIV-1 virion and proviral genome.

(A) The mature HIV-1 virion contains multiple viral gene products. Gag and Env are the main structural proteins that form the spherical shape with spiked glycoproteins protruding from the outer surface. The *gag* gene products include the capsid (Gag_{p24}), matrix (Gag_{p17}), and nucleocapsid (Gag_{p6/7}) proteins. The *env* gene products include surface (SU, Env_{gp120}) and transmembrane (TM, Env_{gp41}) glycoproteins. The Env glycoproteins are inserted in the lipid bilayer, which is derived from the host cell membrane during the budding process. In addition, there are 2 enzymatic (RT and IN) and 3 accessory (Vif, Vpr, Nef) proteins found in the mature virion. HIV-1, like all lentiviruses, contains 2 copies of its single stranded RNA genome (ssRNA). (B) The proviral genome is flanked by 2 long terminal repeats (LTRs) (black). Transcription of viral genes is initiated from the promoter found in the 5'LTR. In the HIV-1 genome, there are 9 open reading frames, which generate 15 different gene products. The Gag-Pol precursor polypeptide is cleaved into *gag* (red) and *pol* (blue) gene products. The Env/Vpu mRNA is singly spliced to produce Vpu (yellow) or the *env* gene precursor (Env_{gp160}) that is processed to Env_{gp120} and Env_{gp41} (purple). Regulatory (Tat and Rev) (orange) and accessory (Vif, Vpr, Nef) (green) proteins are generated by multiply splicing of the Gag-Pol precursor mRNA.

Table 1. Summary of HIV-1 proteins and potential use in VLP vaccines.

PROTEIN	MAIN FUNCTION	VLP ADVANTAGE	VLP DISADVANTAGE	REFS
Gag (CA, MA, NC, p6)	Structural protein Forms sphere of virion, encapsidation of vRNA	Required for particle formation and strong cell mediated immune response	None	192, 310, 447, 459
Env (SU, TM)	Structural protein Virus binding and entry into susceptible cells	Binding and entry of VLPs into susceptible cells, target of neutralizing antibodies	May induce apoptosis of bystander cells	101, 102, 268, 567
Pol-IN	Enzymatic activity Directs proviral integration into host chromosome	Persistent expression of viral proteins, no boosting required (must have LTRs)	Lifelong infection with vaccine strain, may induce reversion or recombination	115, 180, 472
Pol-RT	Enzymatic activity Converts genomic viral RNA to proviral DNA	Additional vaccine target	May induce reversion or recombination, replication is error-prone, escape mutants	257, 276
Pol-PR	Enzymatic activity Cleaves Gag-Pol pr55 into 7 gene products	Cleavage of Gag-Pol and Gag polypeptide into 7 gene products	May induce reversion or recombination, resistance to drug therapy	32, 163
Tat	Regulatory protein Promoting and enhancing viral transcription	Strong cell-mediated response seen early in infection, viral transcription	Strong induction of apoptosis, modulation of expression of many cellular genes	179, 387, 522
Rev	Regulatory protein Nuclear export of unspliced and singly spliced vRNAs	Required for nuclear export of Gag-Pol and Env mRNAs to be translated into proteins	None	273, 522
Nef	Accessory protein Downregulation of CD4 and MHC I, increases infectivity	Very strong cell mediated response early in infection	Downregulates CD4/MHC I, perturbs T-cell activation infectivity of virus	20, 214, 302
Vpu	Accessory protein Downregulation of CD4 and enhances virus release	Inhibits CD4-Env binding in ER and Env degradation, enhances virus budding	Downregulates CD4, lowers expression of Env	5, 175
Vpr	Accessory protein Nuclear localization of PIC, cell cycle arrest (G2)	Additional vaccine target	Induces cell cycle arrest at G2 phase, prevents incorporation of deleterious dUTPs into virion	172, 588, 589
Vif	Accessory protein Required for replication in vivo, enhances infectivity	Additional vaccine target	Restores infectivity in Vif, inhibits cellular antiviral factors	5, 58

I.C.1. Structural and Enzymatic Genes

I.C.1.a. gag

The group associated antigen (*gag*) gene encodes a 55 kDa precursor protein (p55), which is expressed from the unspliced viral messenger RNA (mRNA). The precursor protein is proteolytically cleaved into three main structural gene products that are incorporated into mature virions: matrix, capsid and nucleocapsid. Assembly and maturation of HIV particles is dependent on the Gag gene products.

Matrix

The MA (p17) protein undergoes post-translational myristylation at the N-terminus, promotes attachment of Gag_{p55} to the cell membrane and forms the submembrane layer of the virion. Two distinct features of MA are involved in membrane targeting: N-terminal myristate group and basic residues found within the first 50 amino acids (together known as the “membrane-binding” or “M” domain). Trimeric MA associates with the cell membrane by insertion of the 3 myristate groups into the lipid bilayer located directly above the trimer, and the interaction occurs between the basic residues on MA and bilayer phospholipid head groups. In addition to Gag/Gag-Pol membrane targeting, MA assists in Env incorporation into the virions by association with the cytoplasmic tail of Env^{266, 422}. Although controversial, MA has been implicated in facilitating infection of nondividing cells types (particularly macrophages). MA also contains a nuclear localization signal (NLS) and has been

associated with the pre-integration complex (PIC) and HIV-1 IN^{79, 80, 201, 202}. However, additional studies will need to clarify the role of MA in the infection of nondividing cells.

Capsid

The CA (p24) protein is the most abundant viral protein found in the virus and is required for Gag-Gag multimerization. CA is composed of two domains: 1) N-terminal region or core domain (virion maturation and incorporation of cyclophilin A (CypA)) and 2) C-terminal “dimerization” domain (Gag-Gag interactions). CypA is a peptidyl-prolyl *cis-trans* isomerase, and viruses deficient in CypA exhibit defects early post-infection^{64, 617, 631}. Mutation of the C-terminal third (known as the “interacting” or “I” domain) of CA greatly impairs virus production^{97, 156, 272, 305, 315, 413, 536, 656, 726}. More specifically, mutations in the major homology region (MHR) result in defects in assembly, maturation and infectivity⁴¹³. The MHR forms a network of hydrogen bonds that stabilize the conformation of the entire domain and assists in interactions between Gag molecules^{170, 171, 204, 657}. CA is also required for the incorporation of Gag-Pol polyprotein into virions, which is essential for the recruitment of PR, RT and IN into the virus particle^{281, 602, 612}.

Nucleocapsid

NC is located within the capsid layer and is responsible for packaging of the viral RNA genome⁴⁴². Highly conserved among all retroviruses (except spumaviruses), NC contains 2 zinc-finger motifs (Cys-X₂-Cys-X₄-His-X₄-Cys, CCHC) found in many cellular DNA binding proteins⁴²⁷. NC is tightly associated with the viral RNA in virions by binding to the packaging signal, *psi* (ψ), located near the major splice donor site

(immediately 5' of *gag*)^{107, 366}. The interaction between NC and ψ requires intact zinc fingers and the flanking basic residues^{519, 572}. In addition to RNA binding and encapsidation, NC plays a role in: 1) RNA dimerization^{124,129,558}, 2) Gag-Gag interactions, 3) virus assembly^{155, 272}, 4) tRNA incorporation and annealing to the primer binding site (pbs)/strand transfer during RT^{85, 281}, and 3) stability of the PIC⁹⁰.

p6

In contrast to the other Gag gene products, HIV-1 p6 has not been intensely investigated. This proline-rich protein is located at the C-terminus of Gag and is important in the incorporation of Vpr into the virion^{340, 401, 507}. p6 binds to Vpr at residues 32-39 and three hydrophobic residues in a highly conserved sequence motif (Leu41-X42-Ser43-Leu44-Phe45-Gly46)^{98, 340}. Similar to other retroviruses, HIV-1 p6 contains a “late” or “L” domain, which plays a role in the final release of virions from the cell surface. Mutations in this region (Pro7-Thr8-Ala9-Pro10-Pro11, PTAP) result in the accumulation of virus particles at the plasma membrane²²⁸. In addition, many of these particles have been observed tethered to the membrane, which is suggestive of a block in a very late stage of budding²⁸². However, the role of p6 during assembly/release is still under investigation.

I.C.1.b. *pol*

Three viral enzymes are encoded by the polymerase (*pol*) gene (PR, RT, IN) (Figure 1 and Table 1). The *pol* gene products are derived from the Gag-Pol_{p160}

precursor. This polypeptide is generated by ribosomal frameshifting during translation of Gag_{p55}. The frameshift only occurs 5-10% of the time, thereby ensuring that *pol* gene products are expressed at low levels compared to Gag gene products. All three enzymes are associated with the viral genome.

Protease

PR is responsible for the proteolytic processing of the Gag-Pol_{p160} and Gag_{p55} precursor polyproteins and thus plays a critical role in the maturation of the virion. HIV-1 PR is similar to cellular aspartic proteases such as rennin and pepsin, which contain a conserved sequence (Asp, Thr, Gly; protease amino acid positions 25-27) in the active site¹²⁵. Similar to cellular aspartic proteases, HIV-1 PR uses two apposed Asp residues at the active site to direct a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. Mutation of the Asp, found at amino acid 25, abrogates the activity of PR and prevents the cleavage of Gag and Gag-Pol precursors²⁵. The binding cleft in HIV-1 PR can hold a 7-amino acid peptide⁵⁰. Unlike cellular aspartic proteases, HIV-1 PR functions as a true dimer^{365, 477, 691}. The substrate binding site is located within a cleft formed between the two monomers. The first cleavage event in all retroviral Gag-Pol polyproteins is the autolytic processing of PR. PR is flanked by p6 at the N-terminus and RT at the C-terminus (Figure 1). Autoprocessing of PR appears to occur via 2 steps: 1) intramolecular cleavage at the N-terminus of PR concomitant with the enzymatic activity^{391, 392} and 2) intermolecular cleavage at the C-terminus⁶⁹⁴.

Following its release, PR forms a dimer and cleaves a number of sites in the Gag and Gag-Pol precursors. Gag processing by PR occurs at junctions between MA/CA,

CA/p2, p2/NC, NC/p1 and p1/p6, but each site is cleaved at different rates. Based on Gag proteins translated *in vitro*, the processing occurs at primary, secondary and tertiary sites that are cleaved sequentially in Gag: 1) p2/NC (NC condensation), 2) NC/p6 (formation of submembrane shell), 3) MA/CA (formation of core shell initiation) and 4) CA/p2 (formation of core shell completion)^{511, 639}. Consistent with these results, mutation of the cleavage sites results in particles with aberrant morphologies that is suggestive of a similar order of Gag processing (p2/NC, MA/CA, CA/p2)^{2, 348, 683}. PR appears to be most active just before particle release from the cells³¹¹. Proteolytic cleavage of Gag/Gag-Pol precursors results in a dramatic change in the morphology of the particle, which is known as maturation. Without a functional PR, particles appear doughnut-shaped (immature) by electron microscopy (EM). Premature cleavage of Gag/Gag-Pol polyproteins is detrimental for virion assembly; therefore, it is imperative that PR is not activated until after assembly/budding is initiated.

Reverse transcriptase

RT converts the viral ssRNA genome into the double-stranded (ds) DNA form known as the provirus^{27, 629}. Although each virus contains two strands of RNA, only one provirus is made²⁷⁸. The mature RT holoenzyme is a heterodimer (p51/p66; 250 molecules per virion) and has three enzymatic functions: 1) RNA-directed DNA polymerization (minus-strand DNA synthesis), 2) RNaseH activity (degradation of the tRNA primer and genomic RNA in the DNA/RNA hybrid intermediates) and 3) DNA-directed DNA polymerization (plus-strand DNA synthesis). The polymerase domain is linked to RNase H by a connection domain. The active site contains three critical Asp

residues (110, 185, 186) with two coordinated Mg^{2+} ions. Mutation of these Asp residues abolishes RT polymerizing activity⁹⁵.

Reverse transcription of the HIV-1 ss RNA genome to a ds DNA copy occurs via a series of controlled steps²²⁷. First, minus-strand DNA synthesis is initiated from the 3'OH of the tRNA bound to the pbs, and DNA synthesis continues to the 5' end of the genome. RNaseH digests the RNA portion of the RNA/DNA hybrid leaving the short, ss DNA fragment (minus-strand, strong-stop RNA). The minus-strand, strong stop DNA is transferred to the 3' end of the genome where it binds to the repeated (R) region present at the 5' and 3' ends of the RNA genome. Minus-strand synthesis continues to the pbs at the 5' end of the genome. Meanwhile, RNaseH partially degrades the RNA in the resulting RNA/DNA hybrid. Fragments not removed by RNaseH serve as primers for plus-strand synthesis (major priming site: polypurine tract, PPT). RNaseH removes the tRNA that initially served as the primer for minus-strand synthesis. This exposes the pbs at the 3' end of the plus-strand DNA, allowing the plus-strand DNA to transfer and bind to the homologous region at the 3' end of the minus strand DNA. Plus- and minus-strand syntheses continue to completion. Plus-strand synthesis terminates at the end of the minus-strand at a sequence known as the central termination signal (CTS)⁹⁶. The final product of reverse transcription is a ds DNA molecule that can integrate into the host chromosomal DNA. The high mutation rate of HIV-1 is largely due to the error-prone nature of RT, which lacks proofreading activity and frequently switches templates⁶²⁸. In vivo, the total HIV-1 mutation rate (substitutions, simple deletions, frameshifts and deletions with insertions) was measured at 3×10^{-5} per cycle of replication⁴¹⁴. The mutation rate for HIV-1 was 2-10 fold higher compared to other retroviruses.

Integrase

IN (p32) mediates the integration of the viral DNA into host cell chromosomes during the replication cycle. Retroviral INs are comprised of 3 structural/functional domains: 1) N-terminal zinc-finger-containing domain, 2) core domain and 3) the relatively conserved C-terminal domain. Although the integration of murine leukemia virus (MuLV) was the first described, integration of all retroviruses follow the same series of events^{72, 196}. IN removes 2-3 nucleotides from the blunt 3' terminus of both strands of full-length, ds DNA forming the pre-integration substrate. Randomly, IN catalyzes a staggered cleavage of the cellular target sequence once inside the nuclease. The 3' recessive ends of viral DNA are joined to the 5' ends of the cleaved cellular DNA (strand transfer). Host cell repair machinery fills in the gaps thus completing the integration process^{72, 196, 549}. The integrated viral DNA (provirus) is flanked by a 5 bp direct repeat (5'-TG, CA-3'). The direct repeat is from the duplication of cellular target sequences. Mutation of highly conserved residues, found in HIV-1 IN and other polynucleotidyl transferases (Asp-64, Asp-116, Glu-152 and D,D-35-E motif), block IN function *in vivo* and *in vitro*. Mutating these conserved residues or deleting the gene sequence prevents integration of viral DNA and subsequently replication of the HIV-1.

I.C.1.c. env

The *env* gene encodes for glycoproteins that are important in receptor binding and entry (Table 1)^{11, 168, 541, 652}. The *env* gene is expressed as a polyprotein precursor, Env_{gp160}. Env_{gp160} is then cleaved by a cellular protease, furin, into the two Env

glycoproteins, gp120 and gp41. Env_{gp120} and Env_{gp41} associate through noncovalent interaction. These molecules form a multimeric structure (most likely a trimer) on the surface of the virion^{94, 626, 679, 710}. Env_{gp41} forms the transmembrane domain of the Env complex, while Env_{gp120} is presented on the surface of infected cells or virions. Env_{gp120} has five hypervariable (V) and five constant (C) regions, designated V1-V5 and C1-C5, respectively. The amino acid sequence in the variable loops can vary greatly among HIV-1 isolates^{63, 561, 577, 592, 623, 681, 702, 703}. One such region is V3, which is an important determinant in cell tropism for HIV-1 and contains the chemokine receptor binding domain^{88, 89, 108, 285, 326, 361, 362}.

Both Env_{gp120} and Env_{gp41} are responsible for mediating entry of the virus into host cells. Initially, Env_{gp120} binds to human CD4 (hCD4) on the surface of target cells^{95, 144, 153, 162}. This interaction results in a conformational change in Env_{gp120}, which exposes the chemokine receptor-binding domain. Binding of the virus to the chemokine receptor leads to another conformational change in Env_{gp120} that exposes the fusogenic domain in Env_{gp41}. The fusogenic domain instigates entry of the viral core by fusing the viral and host cell membranes^{95, 191, 263}.

I.C.2. Regulatory Genes

Two viral proteins, Tat and Rev, regulate gene expression of the integrated proviral DNA (Table 1). During the replication cycle, these proteins are expressed very early. Without these proteins, HIV-1 cannot properly undergo viral replication.

I.C.2.a. tat

The *trans*-activator of transcription protein (Tat) is a transcriptional *trans*-activator that greatly improves elongation of viral mRNAs. The HIV-1 5' LTR contains enhancer and promoter regions necessary for the binding of cellular and viral transcriptional factors for viral transcription. In the absence of Tat, the transcription of the integrated proviral DNA is terminated prematurely due to abortive elongation, thus producing short, incomplete transcripts. However, Tat enhances the processivity of elongation and allows for efficient production of full-length HIV-1 mRNAs (Table 1). Tat overcomes the poor processivity of the elongating complexes by binding to the *trans*-activation response (TAR) RNA sequence (stem and loop structure) and is located near the initiation site of the promoter within the 5' LTR. Tat binds to the TAR stem loop, in conjunction with the cellular protein, Tat-associated kinase (TAK). The viral-cellular protein complex phosphorylates the C-terminal domain of RNA polymerase II and converts the transcription complexes into a favorable form suitable for processive elongation. Tat is also secreted from the infected cell. Even though the extracellular activities of Tat are still unclear, Tat inhibits antigen-induced, but not mitogen-induced, proliferation of peripheral mononuclear blood cells (PBMCs)⁶⁵⁵ and stimulates the growth of cultured Kaposi sarcoma cells from patients with AIDS¹⁷⁸.

I.C.2.b. rev

The regulator of virion protein, Rev, is a 19 kDa protein (Table 1). The presence

of Rev in cells is an indication of gene expression switching from the early phase (multiply spliced mRNAs) to the late phase (singly and unspliced mRNAs) of viral transcription. Using cellular post-transcriptional processing machinery, HIV pre-mRNAs undergo a series of modifications (capping, 3'-end cleavage, polyadenylation and splicing) prior to leaving the nucleus. Rev is responsible for the export of viral unspliced and singly spliced mRNA from the nucleus into the cytoplasm. The unspliced mRNA serves as the viral genome or for Gag/Pol expression, whereas Env and Vpu are expressed from singly spliced mRNAs. Rev contains a leucine-rich nuclear export signal (NES) located at the carboxy terminal domain^{187, 409}. The signal is recognized by several cellular proteins such as Crm1, eukaryotic initiation factor 5A (eIF-5A), ribosomal protein L5, exportin 1, and Ran-GTP. These cellular proteins are required for Rev-dependent, nuclear export of viral RNAs. The mechanism by which Rev functions is debatable. The current model suggests that one copy of Rev binds specifically to a sequence in the viral mRNA termed the Rev-responsive element (RRE)⁴¹⁰. Found in the Env_{gp41} sequence, the RRE is approximately 250 nucleotides in length and contains several stem loop structures. The Rev/RNA complex then recruits eIF-5A and ribosomal protein L5. EIF-5a and L5 provide Rev-bound RNA access to exportin-1 and Ran-GTP^{189, 475, 479}. Once the complex is formed, it is translocated to the cytoplasm. The complex disassembles in the cytoplasm by hydrolysis of Ran-GTP to Ran-GDP⁴⁷⁵. Rev is then shuttled back into the nucleus following exposure of its nuclear localization signal.

I.C.3. Accessory Genes

The accessory proteins (Vif, Vpr, Vpu and Nef) have multiple functions within the replication cycle. These accessory proteins are responsible for enhancing viral infectivity and replication. However, they are not always required for replication *in vitro*.

I.C.3.a. vpr

Vpr (viral protein R, 14 kDa) is one of the viral proteins that are incorporated into the virion at high levels (100/virion). There are three major functions proposed for Vpr: 1) stimulation of gene expression by the HIV-1 promoter, 2) transport of the viral PIC to the nucleus following uncoating and reverse transcription and 3) arrest of infected cells in the G₂ phase of the cell cycle. The stimulation of gene expression by Vpr is mediated through interactions with Sp1^{569, 670}, TFIIB³²⁹ and TFIID³²⁹. Following HIV-1 entry and uncoating, Vpr mediates the transport of the PIC to the nucleus. Vpr plays an important role in the ability of HIV to infect nondividing cells, as well as dividing cells, by facilitating nuclear localization of the PIC even in the absence of mitotic nuclear envelope breakdown. In addition to nuclear localization, Vpr also blocks cell division by arresting cells in the G₂ phase.

I.C.3.b. vif

Vif (virus infectivity factor, p23) is incorporated into the virion at small levels, promotes infectivity of particles and is required for reverse transcription of the ss RNA into ds DNA (Table 1). Vif is highly conserved throughout lentiviruses (except EIAV)³¹⁸ and is also essential for replication of HIV-1 in primary lymphocytes, macrophages and some T cell lines. However, multiple cell lines are permissive to *vif*-defective virus (293T, HeLa, COS, SupT1, CEM-SS and Jurkat)²⁰⁰. This discrepancy is most likely due to differences in host cell factors. There are two proposed theories: 1) Vif-permissive cells contain a factor that substitutes for Vif or 2) nonpermissive cells contain a factor that suppresses virus replication in the absence of Vif. Earlier reports suggest a role of Vif as a suppressor of host factor(s) to enhance viral replication, but specific Vif/host protein interactions have not been elucidated^{405, 597, 598}. Vif-defective virions, produced from nonpermissive cells, do not efficiently reverse-transcribe their RNA genomes following infection⁶⁰⁹. The mechanism by which Vif affects reverse transcription is currently being tested.

I.C.3.c. vpu

Vpu (viral protein U, p16) is a multimeric, integral membrane phosphoprotein (Table 1)⁴⁰⁸. Vpu is found at intracellular levels comparable to Gag in infected cells but has not been detected in the virion. The two main functions of Vpu are: 1) enhancement of particle release^{621, 630} and 2) CD4 degradation^{57, 99, 377, 576, 654}. Vpu-defective virus remains at the plasma membrane or in intracellular vesicles but does not affect Gag processing or transport³³³. The mechanism by which Vpu stimulates virus particle

release is still under investigation; however, it is independent of CD4 or Env^{212, 716}. Vpu directly binds to the cytoplasmic tail of CD4 within the endoplasmic reticulum^{57, 99, 377, 576, 654}. This interaction prevents Env binding to CD4, therefore allowing Env to continue to the cell surface⁶⁸⁸. However, the presence of Env is not required for the degradation of CD4 by Vpu^{99, 688}. One model involves CD4/Vpu/h- β TrCP complexes that are targeted for ubiquitin-mediated proteolysis via interactions with the cellular factor, Skp1, and h- β TrCP⁴¹⁵. Inhibition of proteasome activity blocks Vpu-mediated CD4 degradation and supports this theory¹⁹⁵.

I.C.3.d. nef

Negative regulatory factor (Nef, 27 kDa) is the first protein detectable after viral infection and is found at low levels in virions (Table 1). Nef is only found in primate lentiviruses and has several functions including: 1) downregulation of CD4 and MHC class I molecules^{208, 243, 581}, 2) enhancement of virus infectivity and 3) modulation of cellular activation pathways. Nef binds to the di-Leu-based motif in the C-terminal region of CD4, leading to internalization and degradation of CD4 via the endocytosis pathway^{8, 208, 243}. In contrast, downregulation of MHC I by Nef requires the presence of a Tyr-based motif³⁶⁷. This downregulation may occur by increased endocytosis of MHC I from the cell surface or reduced trafficking of MHC I from the trans-Golgi network to the plasma membrane^{72, 236, 367}. The downregulation of CD4 and MHC I may prevent hyperinfection of the cell and/or presentation of viral peptides to the immune systems¹¹⁰. Virus infectivity appears to be enhanced by Nef at an early stage in the replication cycle,

because *nef*-deleted virus fail to efficiently reverse transcribe their genomes after infection^{8, 103, 580}. However, the mechanism by which Nef promotes viral infectivity remains to be elucidated. Several studies have suggested that Nef influences cellular transduction pathways and alters the activation state of the cell⁴¹⁸. This area of research has remained highly controversial due to the differences in cell culture systems, inherent cross-talk between cellular signaling pathways and potential differences between SIV and HIV. Lastly, Nef is proteolytically cleaved by PR; however, cleavage of Nef does not influence its ability to stimulate virus infectivity⁴⁴⁴. Even though Nef is incorporated at low levels in the virion, the majority of Nef found in the virus is smaller than the full-length protein detected in infected cells^{78, 500, 680}. It is not clear at this time what function is served by proteolytic cleavage or virion incorporation of Nef.

I.D. Replication Cycle of HIV-1

The HIV replication cycle occurs in an orderly fashion (Figure 2). HIV-1 entry into target cells is mediated through a complex interaction between the viral envelope glycoprotein and specific cell surface receptors. HIV-1 infects susceptible cells by binding to CD4 on the CCR5 and CXCR4. After coreceptor binding, a subsequent conformational change exposes the fusion domain in Env_{gp41} and results in fusion of the viral and plasma membranes. This process culminates in viral entry and release of the viral core into the cytoplasm of the cell^{95, 144, 153, 160}. The HIV ss RNA genome is transcribed into ds DNA by the virally encoded reverse transcriptase upon successful entry into the target cell^{227, 561, 623} (see Section I.C.1.b). After translocation of the PIC to

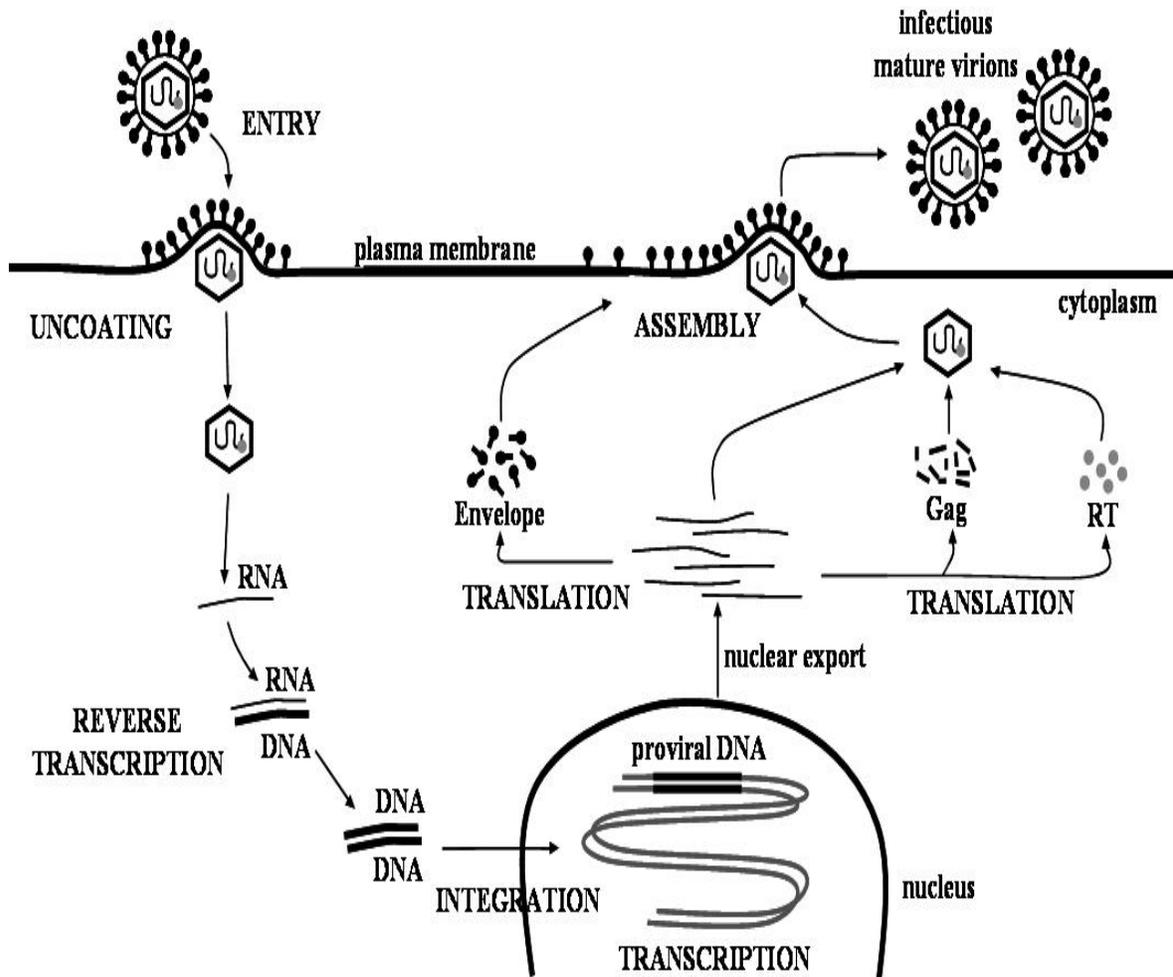


Figure 2. Replication cycle of HIV-1.

HIV-1 enters permissible cells by Env-specific interaction with human CD4 and a coreceptor (CXCR4 or CCR5). The virus fuses to the cell membrane and undergoes uncoating, which results in the loss of the viral membrane. The viral core is then transported to the nucleus. During this time, the viral protein, reverse transcriptase, converts the single-stranded RNA genome to a double-stranded DNA form. The viral genome is transported to the nucleus where it integrates into the host chromosomes via the long terminal repeats (LTRs) and the viral integrase (IN). The integrated, double-stranded DNA form of the viral genome is known as the provirus. Transcription of viral genes occurs via the HIV-1 promoter found in the 5' LTR along with viral and cellular proteins and host cell machinery. Rev is responsible for nuclear export of viral mRNAs. The viral mRNAs accumulate in the cytoplasm and are translated in proteins using host cell proteins and machinery. The viral proteins are then directed to the cell surface where they undergo assembly followed by budding of immature virions. Post-budding,

maturation of the virions causes the particles to become infectious and promotes further infection of other permissible cells.

the nucleus, the viral DNA is integrated randomly into the host chromosomal DNA via the viral integrase and LTRs^{386, 582} (see Section I.C1.b). At this stage, the viral genome is called the provirus. The integrated provirus is flanked by repeat sequences known as LTRs, which are important for integration. In addition, the 5' LTR contains the promoter/enhancer elements necessary for viral gene expression^{109, 386}. Upon cellular activation by environmental and cellular transcription factors as well as the HIV transactivator protein, Tat, transcription of the proviral genome is initiated (see Section I.C.2.a). Using host cell proteins and machinery three different viral mRNAs are produced: 1) multiply-spliced, 2) singly-spliced and 3) unspliced mRNAs^{323, 529, 638}. Initially, the multiply-spliced mRNAs are transcribed during the early phase of HIV-1 transcription (Tat, Rev and Nef). The singly-spliced mRNAs encode for Env, Vpu, Vif and Vpr. Unspliced mRNAs are transcribed in the late phase of HIV-1 transcription (Gag, Gag-Pol, and genomic RNA). Nuclear export of singly and unspliced viral mRNAs is provided by the viral protein, Rev along with cellular proteins and machinery (see Section I.C.2.b). Once in the cytoplasm, these mRNAs are then translated into viral proteins. The envelope proteins are synthesized, glycosylated and processed in the endoplasmic reticulum and Golgi apparatus. Following cleavage by furin, the envelope proteins form multimers (most likely trimers) and migrate to the cell surface^{168, 686}. The structural gene products accumulate at the cell surface and assemble into an immature viral particle, which encapsidates two copies of the viral genome along with the associated proteins. The virus undergoes budding and is released from the infected cell^{192, 310, 459}. In addition to HIV-1 Env, the viral membrane contains host-derived proteins, such as MHC class I¹⁸¹. The particle undergoes a maturation process that involves the

proteolytic processing of the Gag and Gag-Pol polyproteins by the viral protease (see Section I.C.1.b). Gag_{p55} is processed to yield the MA, CA, NC and p6, while Gag-Pol_{p160} is cleaved to produce the Gag gene products plus PR, RT and IN⁵⁸⁷.

I.E. Disease Course and Immune Response to HIV-1 in Humans

There are four major modes of HIV-1 transmission: 1) sexual intercourse, 2) blood products, 3) contaminated needles from intravenous drug use and 4) mother-to-child during the prenatal period¹²¹. Many immunological and viral hallmarks are observed during the course of infection of HIV-1^{106, 114, 122, 501, 633}. The timeline for disease progression from infection with HIV-1 to the development of AIDS varies between individuals. In general, the first symptoms of clinical AIDS become evident 8-15 years after infection.

HIV infection typically follows an established course: 1) primary acute infection often with a mononucleosis-like disease, 2) a prolonged period without obvious, visible symptoms and 3) a severe immunodeficiency that results in the development of opportunistic infections and tumors that lead to the major causes of death in AIDS patients (Figure 3)⁴⁸⁴. The rate of progression through these phases varies among infected individuals. In the first days after infection, the acute phase is characterized by high levels of viral replication in activated lymphocytes located in the lymph nodes (lymphadenopathy). Individuals generally experience flu-like symptoms during this phase of HIV-1 disease (6-12 weeks). During this time, the viral population is relatively macrophage-tropic (M-tropic). M-tropic HIV-1 isolates, seen during the early stage of

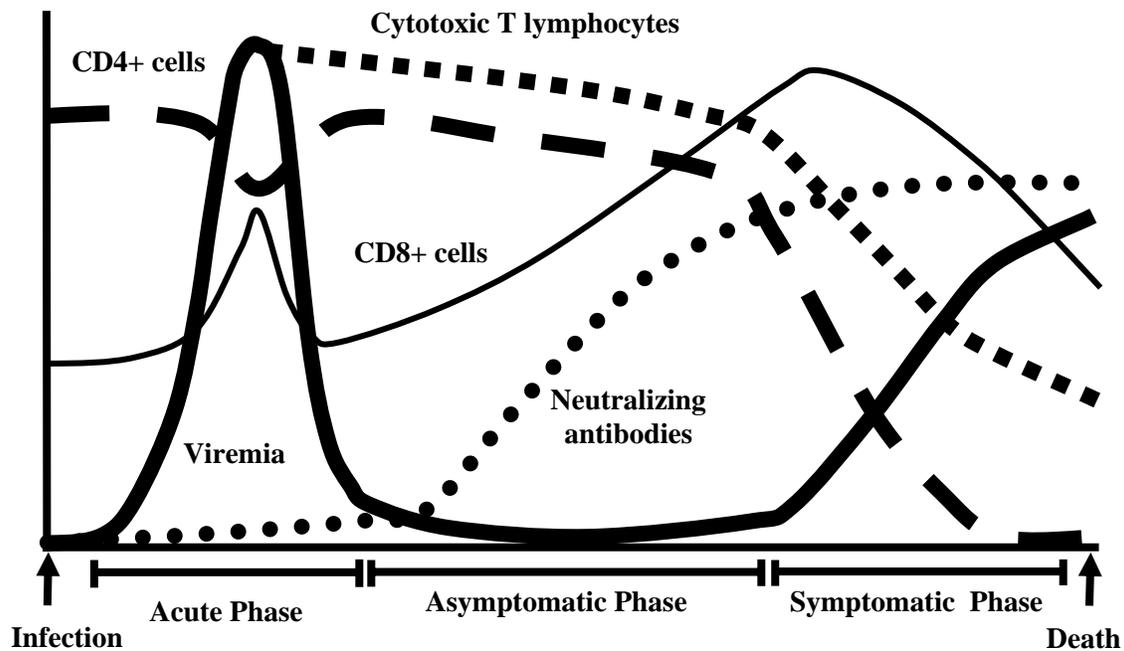


Figure 3. Viral and immunological features during the course of HIV-1 infection.

Initially, an individual becomes infected usually through the exchange of bodily fluids. A transient peak in viremia (———) is noted in the first 2 months post infection. Anti-Gag cellular response is responsible for controlling the initial viremia, which decreases viral loads to low or undetectable levels (set point). The next stage of infection, the asymptomatic phase, can last from months to >10 years. Viral replication persists during this stage, while neutralizing antibody titers gradually increase (●●●). There is a rise in viral titer and concomitantly a dramatic decrease in the number of CD4+ T-cells (- - -). Although the total number of CD8+ T-cells (———) is maintained during this time, the activity of HIV-specific CTLs (■■■) significantly decrease. Once the CD4+ T cell count drops below 200/mm³, the patient develops disease symptoms of disease, is diagnosed with AIDS and usually succumbs to an AIDS-related illness within 2-4 years.

infection, infect cells expressing the chemokine receptor, CCR5. The activation of cytotoxic T cells (CTL) and induction of anti-HIV antibodies result in containment of the initial viremia. An increase in CD8⁺ cytotoxic T lymphocytes is seen in the acute phase, but neutralizing antibodies are not detected until 6 months after infection. CD4⁺ T lymphocytes decrease during this phase but return to near normal levels after six months (Figure 3).

The asymptomatic phase occurs approximately 3-4 months after infection. Minimal viral replication occurs during this stage, and the level of HIV detected in the blood remains relatively stable for many years (Figure 3). The amount of virus in the blood (viral load) decreases to a setpoint (steady state level of virus) and is prognostic for the course of infection and disease; higher setpoints correlate with a more rapid disease progression. Viral load set points of $< 10^3$ copies of viral RNA/mm³ of plasma generally are associated with a slower progression to AIDS^{439, 440, 570}. During the asymptomatic phase, patients experience mild symptoms that may include fatigue, weight loss and shingles. Despite the immune response to HIV-1, virus replication continues at a low rate. At the beginning of the asymptomatic phase, the viral population consists mainly of M-tropic strains. However, the viral population becomes more heterogeneous (M-tropic, dual tropic and T cell tropic (T-tropic) HIV-1 strains) towards the end of the asymptomatic stage.

The symptomatic or AIDS phase is the end stage of HIV-1 disease and is characterized by a dramatic drop in CD4⁺ T lymphocyte population (< 200 cells/mm³ of blood) and is associated with a rise in viremia. Normal healthy adults usually have greater than 1.0×10^3 CD4⁺ T cells per mm³ of blood^{121, 183, 439, 440, 570}. In the lymph

nodes, HIV-1 replication increases and lymphoid cells and tissue are destroyed. In the last phase, the virus population becomes more homogenous with the emergence of the virulent, T-tropic viruses. T-tropic HIV-1 isolates appear later in the course of HIV infection and infect cells expressing the chemokine receptor, CXCR4. The mechanism by which CD4⁺ T cells are depleted remains to be identified. Different mechanisms for the destruction of CD4⁺ and CD8⁺ T cells have been proposed: 1) direct infection of the cell, 2) the induction of apoptosis and 3) syncytium formation of healthy T cells with infected T cells.

AIDS is characterized by a state of immunodeficiency that allows for the development of secondary, opportunistic infections. During this stage, opportunistic infections develop and eventually the patient succumbs to an AIDS-related illness. Some of the most prevalent opportunistic infections of AIDS are *Pneumocystis carinii*, *Cryptosporidium*, *Toxoplasma*, *Mycobacterium avium/tuberculosis* and *Salmonella*^{111, 148, 199, 436, 484, 562}. Tumors usually associated with HIV infection are Kaposi's sarcoma (skin), non-Hodgkin's lymphoma (lymphatic tissues) and primary lymphoma of the brain^{56, 265, 396, 494}.

I.F. Antiretroviral Therapy

Currently, highly active anti-retroviral therapy (HAART) is a treatment regimen widely used by physicians. HAART involves using an assortment of antiretroviral drugs to reduce or prevent viral replication (usually inhibitors of HIV-1 PR and RT). A combination of two or more antiretroviral medications is generally more effective than

using just one of these medications (monotherapy) for treating HIV infection ⁶²⁴. The regimen usually consists of one protease inhibitor (e.g. lamivudine) and one or more reverse transcriptase inhibitors (e.g. zidovudine or stavudine) and results in reduced levels of virus (<50 copies of viral RNA/mm³ blood) after one year of treatment in approximately 60-80% of patients ²⁷⁰. The use of HAART has enhanced both the longevity and quality of life for infected individuals by controlling viral replication ²⁷⁰. Some of the advantages of combination antiretroviral drug therapy for the treatment of HIV are: 1) minimal incidence of HIV-related complications, 2) decrease in viral loads/induction of lower viral setpoints, 3) lessened severity and delayed onset of symptoms and 4) prolonged survival of infected individuals ^{269, 498}. Despite the effectiveness of HAART, several drawbacks are accompanied with this treatment that limit its worldwide use (particularly in developing nations). First, HAART does not protect patients against initial infection nor does HAART clear viral infection. Other disadvantages include: toxicity, non-adherence, lack of efficacy, interactions with other drugs and food, unfavorable pharmacokinetics, transportation and storage, high production cost and drug resistance ^{133-135, 251, 619}.

I.G. Correlates of Protection

The correlates of protection for HIV-1 have yet to be fully elucidated. However, a significant amount of information regarding the mechanism of protection has been accumulated through vaccination trials and natural infection of HIV-1. Live-attenuated virus and Env subunit (gp120 and gp160) vaccines were the first approaches attempted to

generate a vaccine for HIV-1. Due to safety concerns, second generation vaccines, such as inactivated virus, were later developed. Although these vaccines were safer than live-attenuated virus, inactivated HIV-1 was not as immunogenic as the replication-competent virus. The third generation of HIV vaccines included purified or synthetic proteins, which also did not achieve the level of immunity observed with live-attenuated virus. Most recently, a fourth generation of HIV vaccines (DNA and viral vector vaccines) has emerged due to modern advances in molecular biology and genetic engineering. Although most vaccine approaches to HIV have failed to protect the vaccinee from virus challenge, a significant amount of knowledge has been uncovered regarding the mechanisms underlying the generation of immune responses and the development of advanced technologies to monitor the immune response elicited by a vaccine.

As expected, the immune response elicited varies considerably depending on the nature of the immunogen. In general, live-attenuated virus vaccines induce immune responses similar to those observed in natural infection. Killed virus vaccines and purified synthetic proteins preferentially elicit neutralizing antibodies and CD4⁺ T cell responses but not CTLs. Replication defective virus-based vectors, alone or in combination with DNA, induce CTLs and CD4⁺ T cell responses but are less effective in generating neutralizing antibodies. In contrast to HIV-1, a number of live-attenuated virus, inactivated virus or soluble protein vaccines have been proven effective against a variety of viral pathogens (smallpox, measles, polio, varicella zoster, hepatitis B). However, the mechanism of control (antibodies, CD4⁺ T cells, CTLs or combination) varies between pathogens. There are considerable obstacles that have impeded the development of an effective vaccine against HIV-1 including integration of viral

genome/life-long infection of host, viral escape from neutralizing antibodies and CTLs, sequence variability/error-prone nature of HIV-1 RT and immune evasion.

Despite the evasive nature of the virus, both arms of the immune response are directed against multiple viral proteins during a natural infection of HIV-1. Different components of the immune system are effective in producing antiviral responses to different forms of HIV-1 (virions, latently infected cells and virus-producing infected cells). Neutralizing antibodies are efficient in blocking virus particles from infecting new cells but are poorly effective against cell-associated virus. Some CTLs are effective against virus-producing infected cells but not against free virus particles. Neither antibodies nor CTLs are effective against latently infected cells.

Cellular responses to HIV-1 have been studied extensively in infected individuals. There is an abundance of HIV-specific CD4⁺ T cell that secrete IFN- γ despite the loss of HIV-specific CD4⁺ T cells with the capacity to proliferate ⁵¹⁶. This suggests that the problem is not a lack of HIV-specific CD4⁺ T cells but a distortion toward one functional population of CD4⁺ T cells. The cellular response to HIV-1 is ineffective compared to other viruses such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) ^{19, 70, 93, 256}. There are three distinct functional T cell populations that are associated with different conditions of antigen persistence and antigen load during infection with EBV or CMV: CD4⁺ T cells that secrete IL-2 only (antigen clearance), IFN- γ only (antigen persistence and high antigen load) and a combination of IL-2 and IFN- γ (prolonged antigen exposure and low antigen load). Interestingly, HIV-1 long term non-progressors (LTNP) maintained polyfunctional CD4⁺ T cell responses similar to CMV and EBV.

Overall, the same generalities can be assessed to CTLs. Despite a high frequency of HIV-specific, IFN- γ -secreting CD8⁺ T cells that recognize multiple epitopes throughout the viral genome, viral replication is not controlled^{6, 45}. Distinct differences, similar to those seen for CD4⁺ T cells, have been observed in the antigen-specific, CD8⁺ T cells induced against HIV compared to CMV and EBV. The presence of virus-specific CD8⁺ T cells that are able to proliferate and secrete IL-2 seems to be associated with low levels of viral load and viral control⁴⁴³. These effective cells are found in CMV and EBV infections in addition to HIV-1 LTNPs. In general, chronic, progressive HIV-1 infection is associated with a monofunctional T-cell response (IFN- γ), whereas LTNP induce a polyfunctional cellular response from memory CD4⁺ and CD8⁺ T cells at different stages of differentiation²⁵⁶. These results suggest that the effectiveness of the cellular immune response is based on the quality, not quantity, of the vaccine-induced T cells.

Results from several studies have validated this theory. 1) Virus-specific CD8⁺ T cells responses have been detected in individuals exposed to HIV-1 but remaining uninfected^{316, 317, 553}. 2) Following interruption of HAART in HIV-infected patients, virus control is associated with the preservation of HIV-specific CD4⁺ T cell responses during primary infection⁵⁴⁶. 3) In the SIV/maaque model, depletion of CD8⁺ T cells results in uncontrolled viral replication; SIV replication is quickly controlled following the restoration of CD8 + T cell responses^{297, 573}. 4) There is a small group of individuals (LTNP) that does not experience disease progression even in the absence of antiviral treatment. 5) LTNPs maintain polyfunctional, memory cellular responses similar to the

responses observed in CMV and EBV ^{443, 557}. In light of these observations, HIV-specific cellular responses may contribute to the effective control of replication during infection.

In addition to an effective cellular response, a broadly cross-reactive humoral response will most likely be required to protect individuals against viral challenge. Neutralizing antibodies may play a more influential role in preventing the initial infection of HIV rather than in the control of chronic, established HIV infection. Passive immunization with neutralizing antibodies prevented the establishment of chronic infection of chimpanzees with HIV-1 ^{176, 420}. Although controversial, there is evidence that neutralizing antibodies may influence the level of chronic steady-state viremia ⁵⁷⁴. The limited effect may be due to the rapid escape of virus from neutralizing antibodies observed in infected individuals ^{538, 678}.

In the presence of pre-existing, vaccine-induced immunity, the correlates of protection may not be the same as those involved in preventing disease progression during natural infection with HIV-1. Research will need to further define the mechanism of viral control and how pre-existing immunity may affect the outcome of HIV-1 infection. Nonetheless, an ideal HIV/AIDS vaccine will most likely need to elicit both cross-reactive, neutralizing antibodies and a strong cellular immune response against multiple HIV antigens to protect individuals against viral challenge.

I.H. HIV-1 Vaccines

Over the past two decades, many strategies have been explored in the search for an effective vaccine against HIV-1. Several of these vaccines are effective in inducing

strong cellular and humoral response. Some of the most prominent immunogens include: 1) live-attenuated virus, 2) subunit protein, 3) DNA, 4) viral vectors and 5) virus-like particles. Particle-based vaccines (live-attenuated virus and VLPs) are described in detail in Section I.I.

I.H.1. Subunit Vaccines

Unlike hepatitis B, subunit vaccines have not proven effective against immunogens such as HIV-1. Subunit vaccines involve inoculation of purified, soluble protein that induces mainly a humoral response regardless of the inoculation site. Historically, subunit vaccines for HIV focused on the Env protein, although other proteins have been tested. Env is the only realistic target for neutralizing antibodies against HIV-1, because it is the only viral protein found on the outer surface of the virion and mediates entry of the virus into susceptible cells. In early Env subunit vaccine studies in mice and non-human primates, neutralizing antibodies were enhanced^{248, 435} and some (not all) chimpanzees were protected from infection following challenge with HIV^{41, 74}. Based on these results, recombinant Env_{gp120} was evaluated for safety and immunogenicity in humans. Antibodies to Env were detected in almost all vaccinees, and neutralizing antibodies were detected in the majority of recipients^{150, 579}. It became evident later that these neutralizing antibodies were transient and limited to homologous, laboratory-adapted HIV strains^{421, 696}. HIV Env subunit vaccines do not generally induce significant CTL responses against Env^{233, 433}, nor do they neutralize primary isolates^{231, 421, 426}. Most experts in the field do not support monomeric Env_{gp120} as a viable vaccine

candidate for the future^{83, 457}. In spite of the compounding negative results for Env_{gp120} subunit vaccines, there is currently an ongoing Phase III trial testing two bivalent Env_{gp120} vaccines in humans (Vaxgen)^{42, 190, 371}.

The failure of Env_{gp120} subunit vaccines prompted researchers to investigate strategies that may produce more immunologically relevant antibodies to the native structure of Env such as oligomeric gp140 and particle based-vaccines. Various approaches to construct soluble, trimeric forms of Env that more closely mimic the native Env_{gp160} on the surface of virions have been employed. Some of these include stabilized Env_{gp120/gp41} subunits⁵¹, Env_{gp160} and Env_{gp140} oligomers^{167, 710} and trimeric Env^{495, 548}. Soluble, trimerized Env_{gp140}, unstabilized or stabilized with domains (GCN4, T4 bacteriophage fibritin motifs or by additional disulfide bonding (SOS)), elicited modest levels of enhancement of neutralizing antibody compared to antibody elicited by monomeric forms of Env^{1, 61, 563, 578, 711}. Future studies in primates should determine whether neutralizing antibodies induced by vaccination with these modified Envs are able to confer protection against virus challenge.

I.H.2. DNA Vaccines

DNA (genetic) vaccination delivers the gene encoding for a protein rather the proteins or peptides themselves into a host. These genes are usually expressed from eukaryotic expression vectors, which use transcriptional and translational machinery of the transfected eukaryotic cell to produce the associated protein. Plasmids (circular ds DNA) usually contain a eukaryotic promoter and poly adenylation signal for efficient

transcription of the vaccine gene insert. In addition, most plasmids include a bacterial origin of replication and an antibiotic resistance gene for amplification and selection in media. The gene of interest is molecularly cloned into the expression vector at the multiple cloning site (MCS) located between the promoter and poly adenylation signal.

Nearly every gene of HIV-1 has been vaccinated in the form of DNA (most widely tested: *gag* and *pol* gene products, Env, Tat, Nef). Strong antibody and cellular responses were induced with these DNA vaccines. DNA vaccines are particularly suitable for eliciting strong cell-mediated responses, because they may be expressed and presented by professional APC and directed to the MHC I presentation pathway. The majority of the preliminary studies of DNA vaccines were performed in rodents, and the results appeared promising. However, the immunogenicity of DNA vaccines observed in mice has not transferred into other species. Currently, there are 11 HIV-1 DNA vaccines in human trials ²⁸⁶. All trials (except a study with *tat* only) include *gag* in the vaccine cocktail. Most vaccination regimens consist of multiple genes (*gag*, *pol*, *env*, *nef* and *tat*) given as individual genes or as epitopes/peptides.

DNA vaccines can be inoculated into animals by various routes. Intramuscular (i.m.) (needle injection) and intradermal (i.d.)/gene gun (g.g.) inoculations are the two most common routes of DNA immunization. The primary cell type that expresses the DNA vaccine is different in these two systems. Muscle cells are the primary cell that express the DNA following intramuscular inoculation of DNA vaccines ⁶⁴⁴. Muscle cells are not professional antigen-presenting cells (APCs), so the main function of these cells is to produce protein in large quantities. The proteins are then engulfed by APCs, which transport the immunogen to the regional-draining lymph nodes where the immune

response is initiated^{113, 521}. In contrast, gene gun inoculation results in direct transfection of dendritic cells. Proteins are expressed in these transfected cells and presented on MHC I. In addition, proteins may also be engulfed by other untransfected APCs and presented on MHC II molecules. Both types of inoculation lead to cellular and humoral immune responses.

Several methods have been implemented to increase the immunogenicity of DNA vaccines: the addition of strong transcription and translation enhancers into the vaccine vector³⁴⁶, codon optimization of gene sequences^{18, 253, 485}, and the use of adjuvants (interleukin (IL) 12 (IL-12), RANTES, IL-2)^{29, 221, 583, 706}. In particular, codon optimization of gene sequences has greatly enhanced the immunogenicity of DNA vaccines. The use of codon optimization and adjuvants are further described for particle-based vaccines in Section I.I.3.g.

The most effective means of augmenting the immune response by DNA vaccines is the inclusion of DNA as the priming component in a heterologous “prime-boost” vaccination regimen. Prime-boost vaccination strategies consist of 2 different vaccine vectors encoding a common immunogen. This combinatorial approach usually results in an enhancement of the immune response compared to either immunogen alone^{28, 382}. Some of the boosting components that have been used in conjunction with DNA vaccines are viral vectors, subunit proteins and purified particles^{15, 252, 254, 292, 527, 543, 575}.

I.H.3. Viral Vector Vaccines

Vaccination of live viral vectors expressing HIV proteins has demonstrated

favorable immunogenicity data in rodents and non-human primates. In general, a viral vector is used to deliver the gene of interest into a susceptible cell. The viral vector is either attenuated (replicates but does not cause disease in host) or undergoes only a single cycle of replication in the host. Once the viral vector infects the cell, transcription of the HIV gene(s) located within the genome of the viral vector can be initiated. Therefore, proteins expressed from viral vectors may elicit humoral and cellular responses.

Several viral vectors have been tested in rodents and primates containing genes from many pathogens. Although vaccinia virus may be an effective vector for HIV genes, safety issues preclude its use in humans due to a case where vaccinia virus disseminated and caused a fatal encephalitis in an immunosuppressed, HIV-infected individual⁵³⁵. Therefore, the majority of research using poxviruses as vectors for HIV research involves an abortive replication cycle in human cells. Recombinant modified vaccinia Ankara (MVA), canarypox and fowlpox (FPV) produce sufficient HIV proteins to induce cellular and humoral responses against multiple genes in non-human primates. Currently, there is a human trial in Thailand testing the efficacy of a canarypox vector expressing HIV Env, but the results to date do not look promising¹⁸². In addition, there are early-phase human clinical trials with HIV proteins expressed from recombinant MVA and FPV that are still ongoing.

In contrast, recombinant adenovirus vectors have become a promising AIDS vaccine candidate⁵⁹⁴. Adenovirus 5 (Ad5) was made replication-incompetent by deletion of the E1 and/or E3 gene(s). In rodents and non-human primates, this recombinant viral vector has proven highly immunogenic as a vector for HIV proteins⁵⁹⁵. These vaccines are currently entering advanced-phase human clinical trials. So far, HIV-

specific T cell responses have been detected in the vaccinees. However, there are serious problems associated with pre-existing immunity to the vector that may limit its success in humans³⁰. Multiple strategies have been developed to circumvent this problem. One of these approaches involves a prime-boost strategy incorporating DNA expressing the same immunogen prior to vaccination with the recombinant Ad5³⁷⁹. In addition, research is underway to vaccinate volunteers with different serotypes of human or chimpanzee adenoviruses expressing HIV gene products, thus avoiding the issue of pre-existing immunity to Ad5. Some other live recombinant viral vectors currently being testing include: 1) Venezuelan equine encephalitis (VEE) virus, 2) Semliki forest virus (SFV) and 3) parvovirus adeno-associated virus (AAV).

The use of a prime-boost vaccination regimen has recently become quite popular for lentiviruses. Viral vectors have become a key component of these strategies due to their success in primates. Although viral vectors and DNA elicit cellular immune responses quite efficiently, a protein component will need to be incorporated in the vaccination regimen to induce antibodies capable of neutralizing a broad range of viruses.

I.I. Particle-Based HIV-1 Vaccine Therapy

I.I.1. Introduction

The use of live-attenuated viruses as vaccines has been successful for the control of viral infections. However, the development of an effective vaccine against the human immunodeficiency virus (HIV) has proven to be a challenge. HIV infects cells of the

immune system and results in a severe immunodeficiency. In addition, the ability of the virus to adapt to immune pressure and the ability to reside in an integrated form in host cells present hurdles to vaccinologists to overcome. A particle-based vaccine strategy has promise for eliciting high titer, long-lived, immune responses to a diverse number of viral epitopes from different HIV antigens. Live-attenuated viruses are effective at generating both cellular and humoral immunity. However, a live-attenuated vaccine for HIV is problematic. The possibility of a live-attenuated vaccine to revert to a pathogenic form or recombine with a wild-type or defective virus in an infected individual is a drawback to this approach. Therefore, these vaccines are currently only being tested in non-human primate models. These vaccines are effective in stimulating immunity, however, challenged animals rarely clear viral infection and the degree of attenuation directly correlates with the protection of animals from disease. Another particle-based vaccine approach for HIV involves the use of virus-like particles (VLP). VLPs mimic the viral particle without causing an immunodeficiency disease. HIV-like particles (HIV VLP) are defined as self-assembling, non-replicating, nonpathogenic, genomeless particles that are similar in size and conformation to intact virions. A variety of VLPs for both HIV and SIV are currently in pre-clinical and clinical trials.

I.1.2. Live-Attenuated Virus Vaccines

Historically, live-attenuated vaccines have been widely used for the control of many viral infections such as measles, smallpox, mumps, and rubella^{157, 304}. The use of live-attenuated virus therapy has been an attractive vaccine strategy because it: 1) elicits

both humoral and cell-mediated immune responses, 2) is capable of eliciting an immunological memory response, and 3) often only requires one or two immunizations to elicit high titer protective immunity. For more information on live-attenuated vaccines see reviews by Ruprecht, Johnson, Geretti, Kuwata and Haga.^{213, 247, 299, 358, 556}

The effective use of live-attenuated lentiviral vaccines has demonstrated that these formulations are effective at eliciting protective immune responses in a non-human primate model²⁹⁹. Live-attenuated vaccines elicit a robust, broad CTL response in conjunction with high levels of cross-reactive neutralizing antibodies²⁹⁸. Moreover, live-attenuated lentiviral vaccines persistently express viral antigens requiring fewer boosts, contain multiple viral antigens including the native Env conformation(s), and are capable of infecting professional antigen presenting cells (APCs). However, research using live-attenuated HIV vaccines have been limited to non-human primates due to: 1) the potential of the attenuated virus to revert to a virulent form, 2) the possible recombination of the vaccine strain with wild-type, pathogenic virus in an infected individual, 3) the ability of the proviral genome to integrate into the host genome, 4) the dysregulation of the immune system by viral proteins and 5) disease caused by the vaccine strain. In addition, live-attenuated lentiviral vaccines have an inverse relationship between attenuation and efficacy as observed in multiple rhesus macaque studies, therefore as the degree of attenuation increases the efficacy of the vaccine decreases^{143, 299, 300}.

I.I.2.a. Live-attenuated SIV vaccines

The SIV/rhesus macaque model has been used extensively to study the efficacy

and safety of potential HIV-1 vaccines^{143, 217, 616}. Some of the early attenuation strategies focused on abrogating the function of Nef. A representative study achieved attenuation of SIV_{mac}C8 by deleting 4 amino acids in *nef*. The 12 base-pair deletion (amino acids 143-146 in *nef*) is found in the region where *nef* and the 5' LTR overlap⁶⁸². Interestingly, 17 weeks post-vaccination, the vaccine strain had reverted to a pathogenic form and the monkeys developed AIDS-like disease. Virus isolated from the PBMCs of these vaccinated monkeys contained a functional Nef protein and upon further analysis, the deleted gene sequences were restored. Similar results were seen with a live-attenuated SIV_{mac}239 vaccine containing a single base-pair mutation that introduced a premature stop at amino acid 93 in Nef³²⁴. Consequently, attenuated SIV vaccines with deletions in *nef* require additional attenuation to further debilitate the virus and prevent reversion of the vaccine strain to a wild-type, pathogenic entity.

Multiple mutations were introduced into SIV vaccine strains to further debilitate the virus. Two attenuated SIV strains, SIV_{mac}293 Δ *nef* (SIV Δ *nef*) containing a complete deletion of the *nef* gene and SIV_{mac}293 Δ 3 (SIV Δ 3), containing a combination of three deletions including the *nef* and *vpr* genes and the negative regulatory element (NRE) of the LTR were constructed^{218, 453}. Deletion of the *nef* and *vpr* genes resulted in an attenuated strain of SIV that does not achieve high levels of virus replication and does not lead to development of disease in rhesus macaques. The NRE of the LTR was removed to ensure adequate replication of the vaccine strain. Twenty-four adult rhesus macaques were vaccinated (intravenously, i.v.) with SIV Δ *nef* or SIV Δ 3 (12 monkeys each group). Seventeen animals developed persistent infection with the vaccine strain and had long-lasting anti-Env and anti-Gag antibodies. In contrast, 7 monkeys were only transiently

infected and they did not mount persistent antibody responses. The macaques were divided into 4 groups and challenged at different intervals (weeks 8, 20, 79, or unchallenged) with wild-type, pathogenic SIV_{mac251} (10 animal infectious doses). After challenge, the 17 monkeys (with persistent immune responses) vaccinated with SIV Δ nef or SIV Δ 3 showed no signs of simian AIDS compared to naïve monkeys (week 79). The animals receiving SIV Δ 3 elicited a better immune response compared to macaques immunized with SIV Δ nef as determined by cell-associated viral loads, CD4⁺ cell counts, and disease symptoms. However, there were no CTL assays performed in this study. Initial safety and efficacy studies with SIV Δ 3 appeared promising in adult macaques^{123, 699}. However, further analysis revealed that this vaccine caused an AIDS-like illness in adult macaques and death in neonatal macaques at high doses^{23, 24}. Therefore, it is conceivable that a live-attenuated HIV-1 vaccine based on similar attenuation strategies may result in AIDS in vaccinated patients.

In contrast to initial studies with SIV Δ 3, Wyand *et al.* showed neonatal rhesus macaques to be resistant to SIV Δ 3⁷⁰⁰. Neonatal macaques did not develop AIDS-like symptoms upon exposure to SIV Δ 3 from pregnant mothers during gestation or delivery. Adult female macaques were vaccinated (i.v, 11.3ng of Gag_{p27} in the second trimester between days 80-100), and the offspring were orally vaccinated (5, 50, or 283ng of Gag_{p27}) on the day of delivery. Eighty-nine percent of the neonatal macaques were successfully vaccinated with no adverse effects. However, 10% of the neonatal monkeys developed high levels of viremia and subsequent AIDS-like disease. Interestingly, these two animals were born to the unvaccinated mothers and therefore these neonates lacked the associated maternal antibodies that the other uninfected neonates possessed. In

addition, there was a lack of *in utero* transmission of SIV Δ 3 to the neonates born to vaccinated mothers.

Another interesting aspect of attenuated lentiviruses is that the degree of attenuation inversely correlates with ability to elicit effective immune responses. Johnson *et al.* demonstrated that SIV strains with varying degrees of attenuation differed in their ability to elicit high titer immune responses in rhesus macaques³⁰⁰. Female monkeys were vaccinated intravenously with one of the following attenuated SIV_{mac}239 strains: SIV Δ 3, SIV_{mac}293 Δ 3X (SIV Δ 3X), or SIV_{mac}293 Δ 4 (SIV Δ 4). The SIV Δ 3X vaccine strain contains a complete deletion of the *nef* and *vpx* genes that are important in achieving high levels of virus replication. In addition, the negative regulatory element (NRE) in the upstream (US) sequences of the LTR was removed to ensure adequate viral replication of the vaccine strain. SIV Δ 4 has the same three deletions as SIV Δ 3X plus the complete deletion of the *vpr* gene. Rhesus macaques were then challenged (61 weeks post vaccination, p.v.) with pathogenic SIV_{mac}251 (100ng (i.v.) or 48ng (vaginally) of Gag_{p27}). Monkeys immunized with any of these vaccines and challenged with live virus had low levels of viremia and normal CD4⁺ cell counts. Thirty-three percent of macaques vaccinated with SIV Δ 3X and 50% of macaques vaccinated with SIV Δ 4 were also superinfected with the challenge virus. After challenge, vaccinated monkeys had lower viral loads than challenged naive animals. The most likely reason for the ability of the vaccinated monkeys to survive challenge was the induction of an early CTL response directed at epitopes in the Gag protein and not neutralizing antibodies against Env.

Live-attenuated SIV vaccines have also been reported to induce cross-clade viral protection^{354, 483, 698}. Wyand *et al.* vaccinated rhesus macaques with SIV Δ 3 and found

them to be resistant to a challenge with highly pathogenic SHIV_{89,6P} or pathogenic SIV_{smE660}⁶⁹⁸. Monkeys immunized with SIV Δ 3 and challenged intravenously with SHIV_{89,6P} (37 months p.v.) had normal CD4⁺ T cell counts and were free of AIDS-like disease even though all the monkeys had fluctuating viremia (300-10,000 copies of RNA/ml of plasma). Other monkeys vaccinated with SIV Δ 3 and challenged with SIV_{smE660} had lower levels of viremia compared to naïve animals. However, the levels of viremia in SHIV_{89,6P} challenged monkeys were significantly lower than the viremia in monkeys using SIV_{mac239} and significantly less compared to previous studies with SIV_{mac251}^{123, 299, 698}.

Overall, the results from these live-attenuated SIV studies revealed that a broadly cross-reactive, long-lived immune response can be elicited in non-human primates after vaccination. However, the variability in attenuation (and reversion capability) inversely correlated with the efficacy of the vaccine. Therefore, similar outcomes and complications could be expected using live-attenuated HIV strains in humans.

I.1.2.b. Live-attenuated HIV vaccines

HIV-1 infection results in a severe immunodeficiency in only humans and chimpanzees; each syndrome has similar epidemiology and disease outcomes^{463, 488}. The HIV/chimpanzee model has been used to study HIV pathogenesis and vaccine design^{220, 321}. However, in 1998, the U.S. National Institutes of Health (NIH) implemented a chimpanzee-breeding moratorium on NIH-supported AIDS research thereby curtailing almost all HIV/AIDS vaccine studies using chimpanzees. Currently, there are no

sponsored studies involving live-attenuated HIV vaccines in human subjects by the NIH or the World Health Organization (WHO). In this section, live-attenuated viruses will be summarized using a cohort of individuals unintentionally infected with an attenuated strain of HIV.

Researchers have been able to explore the efficacy of live-attenuated virus vaccination in individuals unintentionally infected with attenuated strain(s) of HIV via contaminated blood products (1981-1984) from a common infected donor. This study is known as the Sydney Blood Bank Cohort (SBBC) ^{369, 537}. Nine individuals were given blood products infected with an attenuated strain of HIV-1, which contained a 3' end deletion of the *nef* gene ¹³⁰. This attenuation was similar to the SIV Δ nef vaccine described previously. One member of the SBBC, who had systemic lupus erythematosus, died in 1987 (at 22 years old) of causes possibly related to HIV infection, while two other recipients have since died of causes unrelated to HIV.

Three members of SBBC are long-term non-progressors (LTNP) and continue to maintain near normal CD4⁺ T lymphocyte counts and have minimal HIV/AIDS symptoms ^{166, 370, 434, 724}. Each LTNP has maintained robust HIV-specific proliferative and CTL responses to Gag_{p24}. In contrast, the original donor, as well as two recipients, have dramatically declining CD4⁺ T cell counts, detectable viral loads, and are now termed long-term survivors (LTS). Two of the LTS members (D36 and C98) were re-evaluated in 2002 before HAART therapy was initiated ⁵². Before therapy, both members had low CD4⁺ T cell counts (160 and 387/mm³ blood) and high viral loads (9900 and 11491 copies of vRNA/ml of blood). After one month of therapy, both individuals had a significant drop in the levels of viremia (<400 copies/ml of blood) as

well as a concomitant increase in the number of CD4⁺ T lymphocytes. Virus samples from both members were analyzed. Interestingly, the deletion in the overlapping region between *nef*/LTR was found to be larger than the original documented deletion.

In 1999, the donor developed AIDS, and Jekle *et al.* compared viral strains collected before (1995) and after (1999) the onset of AIDS ²⁹⁶. Both isolates were less effective in depleting CD4⁺ T cells compared to a reference dual tropic strain containing the *nef* gene. CD4⁺ T-cell depletion was assessed by measuring the ratio of CD4⁺ to CD8⁺ T cells in the infected and uninfected individuals. In contrast, virus isolated in 1999 readily induced apoptosis in CD4⁺ T cells in human lymphoid cell cultures compared to the 1995 sample. While the 1995 viral strain was restricted to CCR5⁺ cells, the 1999 isolate could efficiently and equally infect CCR5⁺ and CXCR4⁺ expressing cells. Therefore, conversion from an R5-restricted to an X4-phenotype was correlative with enhanced cytopathic capabilities and advancement to AIDS. Evidence from these studies supports the possible use of a live-attenuated HIV vaccine in humans. However, it also identifies the shortcomings and safety issues associated with this type of vaccine.

I.1.2.c. Live-attenuated SIV-HIV (SHIV) chimeric vaccines

Chimeric SIV-HIV (SHIV) viruses are recombinant SIV containing the HIV-1 envelope glycoprotein. SHIVs have been used as vaccines, as well as challenge viruses, for many years ^{177, 355, 675, 687, 718}. The prototypic SHIV, NM-3rN, encodes the *env*, *tat*, *rev*, *vpu*, and *vpr* gene sequences of HIV-1_{NL4-3} and the long terminal repeats, *gag*, *pol*, *vif*, *vpx*, and *nef* genes from SIV_{mac239} ^{357, 560, 591}. Attenuation of a SHIV strain is usually

achieved by deletion of *nef*, *vpr*, or both genes and usually results in a nonpathogenic strain for macaques^{247, 288}. In contrast, live-attenuated SIV or HIV vaccines often cause disease in primates^{23, 24, 476}. Some advantages of using attenuated SHIV for vaccine development include: 1) use of monkey models to evaluate vaccines, 2) induction of strong and long-lasting cell-mediated responses and humoral immune responses^{288, 289, 375, 658}, 3) the ability to test the efficacy of neutralizing antibodies against HIV-1 Env, 4) cross-reactivity of immune responses to SIV proteins with HIV proteins, 5) replication-competence of SHIVs in human and macaque peripheral blood mononuclear cells (PBMCs)^{398, 560}, and 6) increased safety of SHIV vaccines compared to HIV live-attenuated vaccines^{357, 591}.

Numerous attenuated SHIV vaccines have been constructed^{247, 643, 698} including SHIV-dn (deleted *nef*), SHIV-drn (deleted *vpr* and *nef*) and SHIV-dxrn (deleted *vpx*, *vpr*, and *nef*). All three vaccines are derived from the non-pathogenic paternal strain, SHIV_{NM-3rN}²⁸⁸. Macaques vaccinated with SHIV-dn and SHIV-drn had transient viremia of the vaccine strain, whereas no virus replication was detected in monkeys inoculated with SHIV-dxrn. Overall, all three groups of SHIV vaccinated macaques remained disease-free before challenge. Fifty percent of the macaques immunized with SHIV-dn possessed neutralizing antibodies and CTLs specific for SIV Gag and HIV-1 Env proteins. In addition, most animals demonstrated elevated natural killer cell activity. Following intravenous challenge with SHIV_{NM-3rN} (1 or 2 years p.v.), no signs of integrated genome were found in the plasma, PBMCs, or inguinal lymph nodes two years after vaccination. Macaques immunized with SHIV-dn were completely protected from homologous challenge, whereas animals vaccinated with SHIV-drn (2 of 4) and SHIV-

dxrn (4 of 4) were infected with the challenge strain (detected by PCR). Macaques that were PCR positive (SHIV_{NM-3rN}) maintained the presence of much lower viral loads that were sporadic and delayed compared to naïve animals. Results from this study also support the observation of an inverse relationship between attenuation and efficacy of live-attenuated virus vaccines.

SHIV-dn was then re-evaluated for the ability to protect non-human primates with the highly pathogenic SHIV_{89.6P}¹⁷⁷. Four SHIV-dn vaccinated macaques were challenged with SHIV_{89.6P}. Levels of SHIV_{89.6P} were three to five-fold lower in vaccinated animals compared to naïve macaques. In addition, there was no decline in the number of CD4⁺ T cells in three of the four vaccinated macaques, whereas the CD4⁺ counts in the naïve macaques decreased dramatically. Enhanced levels of protection were observed in macaques immunized with SHIV-dn and challenged vaginally with SHIV_{89.6P}. The vaccinated animals did not develop an immunodeficiency disease even though they were infected with the challenge virus (SHIV_{89.6P}).

Overall, the use of nonpathogenic SHIVs for the development a live-attenuated HIV vaccine is promising, because they are safer than live-attenuated HIV or SIV vaccines. Live-attenuated SHIV vaccines are able to persistently infect monkeys, and most likely humans, in a similar manner to live-attenuated SIV vaccines without causing disease. The animals immunized with live-attenuated SHIV vaccines did not develop disease or die from the reversion of the vaccine strain to a virulent form. Rather, they died from AIDS-like illness because they failed to elicit a protective immune response to the challenge strain. Consequently, the efficacy of SHIV vaccines must be improved before they can be used for human clinical trials.

I.I.3.Lentivirus-Like Particle Vaccines

I.I.3.a. Methods of constructing, purifying and administering VLPs

Virus-like particles (VLPs) or “pseudovirions” can be defined as self-assembling, nonreplicating, nonpathogenic, genomeless particles that are similar in size and conformation to intact virions. For lentiviruses, this definition permits inclusion of VLPs consisting of HIV and/or SIV gene products. VLPs must contain capsid (Gag_{p24} (HIV) or Gag_{p27} (SIV)) proteins to effectively undergo particle assembly, budding and release from the host cell to form a spherical structure encapsidated by a lipid bilayer derived from the host cell. The capsid protein can be expressed as an individual protein or as one of the gene products derived from the proteolytic processing of the Gag precursor polypeptide (Gag_{pr55} (HIV) or Gag_{pr56} (SIV)). Proper cleavage of the precursor polypeptide by PR results in expression of capsid (Gag_{p24}), matrix (Gag_{p17}), nucleocapsid (Gag_{p6/p7}), polymerase (RT), integrase (IN), and protease (PR). Most VLP vaccine strategies involve removing the IN gene along with one or both LTRs to prevent integration of the vaccine into the host genome and/or recombination with live or defective virus in an infected individual.

Multiple combinations of viral proteins may be used to generate VLPs. The Gag-Pol polypeptide precursor, Gag_{pr55}, encodes for structural proteins (p24, p17, and p6/p7) and viral enzymatic proteins (RT, IN, and PR). Protease must be expressed to properly cleave the polypeptide precursor to generate products that will be able to assemble and subsequently bud from the cell forming a particle. Gag_{p24} VLPs contain only the capsid

protein that are capable of self-assembly and budding. VLPs may consist of only Gag proteins (Gag_{p24} only or Gag_{p24} and Gag_{p17}) surrounded by a lipid bilayer derived from the host cell during budding or these particles can also have Env protruding from the lipid bilayer. Some VLPs contain sequences from env inserted into dispensable regions of the gag gene, and therefore they do not contain Env incorporated into the particle. Most VLPs that have been described contain different forms of the Env protein including unprocessed Env (Env_{gp160}), processed Env (full length, Env_{fl}), and truncated Env (Env_t).

Lentivirus-like particles can be constructed and purified by a variety of methods, but most often use a viral expression system such as baculovirus or vaccinia that effectively produce HIV/SIV particles^{81, 210, 245, 666, 714}. The vaccinia expression system consists of infecting cells (TK-143B, human B cell) with wild-type vaccinia virus that are then transfected with plasmids expressing Env and Gag proteins. The recombinant vaccinia virus (rVV), with the gag and env genes incorporated in the viral genome, is selected for resistance using BUdR, β -galactosidase expression (selectable markers), and plaque purification. Recombinant vaccinia infects the TK-143B cells, expresses Gag and Env proteins that assemble and bud from the cells as VLPs. The VLPs are then purified by ultracentrifugation using standard 20-60% sucrose or Optiprep gradients¹⁴⁵. These purified VLPs can then be resuspended in saline and used for vaccination. EM, silver staining of protein gels, and immunoblots can validate purity of the preparations.

Baculovirus expression systems are faster and generate a higher yield of virus for VLP production compared to vaccinia expression systems⁶⁶⁶. Insect cells (Sf9) are directly transfected with recombinant baculovirus (rBV) DNA that contains the genes of interest (gag and env). Negative selection against non-rBV is usually achieved using

gancyclovir. The rBV infects Sf9 cells and results in expression of Gag and Env proteins that assemble and bud from the cells as VLPs. Plaque purification of rBV is not required in this system. The method and confirmation of purification of VLPs in this system is similar to the vaccinia expression system.

Lentivirus-like particles can be administered to rodents or primates as ex vivo, purified particles or as DNA plasmids expressing the gene products to form VLPs in vivo. Previous studies have been able to elicit mucosal and systemic immune responses using VLPs. Mucosal immunity is often elicited by inoculating particles in saline directly onto mucosal surfaces. Intravenous (i.v.), intraperitoneal (i.p.), intradermal (i.n.), or intramuscular (i.m.) immunization of DNA plasmids or purified particles in saline or DNA coated onto microparticles and administered by high pressure (gene gun) can elicit predominately systemic but also mucosal immune responses.

1.1.3.b. Immunogenicity of virus-like particle vaccines

A major advantage of a VLP approach compared to live-attenuated virus is a VLP expresses multiple viral epitopes that stimulate a diverse set of immune responses without many of the deleterious effects of a live-attenuated virus. VLPs have the potential for activating both the endogenous and exogenous antigen pathways leading to the presentation of viral peptides by MHC class I and class II molecules. These multi-epitope vaccines are more likely than their single component counterparts to generate a broad-based immune response capable of clearing HIV-1 escape mutants. Moreover, one vaccine expressing VLPs may be more cost efficient than co-inoculating multiple single

gene vaccines for future phase I clinical trials. An advantage of VLPs compared to single recombinant protein vaccines is the ability of VLPs to bind and enter cells expressing appropriate receptors. Env-mediated entry of the HIV-like particles are able bind to CD4 and chemokine receptors via gp120 and enter into professional antigen presenting cells such as macrophages and dendritic cells (both cell types express CD4 and CCR5). After infection, viral proteins can be processed and presented on MHC class I molecules, therefore promoting presentation to T-cells by APCs. In addition, cell-free VLPs bound with antibodies can be taken up by phagocytic cells via Fc receptors, thus increasing MHC class II presentation.

Antigens expressed in their native conformational form can elicit more effective responses compared to proteins in their non-native forms ⁵⁶³. Many neutralizing antibodies directed against HIV are elicited against conformational epitopes only present in the native form of Env, and some are only exposed after binding to CD4 during entry ^{84, 455, 456, 489, 503, 504, 518, 702}. Many Env_{gp120} monomeric protein vaccines elicit high titer anti-Env antibodies. However, these antibodies often do not neutralize primary isolates of HIV ^{28, 33, 84, 232, 421, 649, 650, 710}. In contrast, Env presented as a native trimer conformation more effectively elicits neutralizing antibodies. Env_{gp160}, expressed from DNA plasmid, undergoes normal processing and glycosylation and is incorporated on the surface of the transfected cells. Soluble Env_{gp140} engineered to maintain a trimerized conformation has also been shown to elicit cross-reactive neutralizing antibodies and therefore these results further support the use of native Env structure(s). VLPs expressing native forms of Env have the potential to elicit a strong neutralizing antibody response to Env and may lead to enhanced ADCC. Particle-based vaccines, containing

native forms of Env, in addition to other viral antigens have the potential to induce strong humoral and cell-mediated responses to multiple viral proteins. However, particle-based vaccine strategies are still in their infancy and future studies need to be conducted to determine the optimal vaccination regimen, which includes the form of the antigen, delivery system, and the use of adjuvants to induce high titers of neutralizing antibodies and strong cell-mediated responses.

I.I.3.c. DNA vaccines expressing virus-like particles

Multiple studies have demonstrated the effectiveness of lentivirus-like particles expressed from a DNA vaccine. A DNA vaccine expressing a SIV VLP (pIV) was constructed to mimic a naturally-occurring attenuated viral strain of SIV (SIV_{smB7})⁶⁰⁷. pIV expresses an SIV VLP that is attenuated due to a deletion in the genome that deletes sequences that encode for IN, *vif*, *vpx*, and most of *vpr* (~1.6bp deletion, 5' end of *pol* to 5' end of *vpr*). In addition, the cytomegalovirus immediate-early (CMV-IE) promoter was substituted for the 5' LTR to drive high levels of transcriptional expression of the VLP genes. The ensuing particles were noninfectious (no integration or RT activity) and nonpathogenic (no disease from vaccine strain). Vaccination of New Zealand white rabbits with the pIV resulted in enhanced anti-Env antibody titers (1:2048) and robust lymphoproliferative responses from isolated PBLs and splenocytes after *in vitro* stimulation with whole virions (SIV_{smB7}) or SIV Env_{gp130}.

Lu *et al.* also constructed plasmids expressing SIV VLPs using the SIV_{mac239} genome⁴⁰⁰. The vaccine, pSIV239.Δ*pol*, generates non-replicating particles.

SIV239.Δpol VLP is derived from the live-attenuated strain, SIVΔnef, and it contains the *gag*, *vif*, *vpx*, *vpr*, *rev*, *tat*, and *env* genes. LTR and *pol* sequences were deleted by site directed mutagenesis/molecular cloning techniques to ensure the vaccine could not revert to a pathogenic form. LTR sequences were removed, a 754bp fragment (nt3571-4325) was deleted, and a stop codon was inserted to render the *pol* gene defective. The *nef* gene was also defective by introduction of a premature stop codon at the 5' end of the 3' LTR. DNA vaccines expressing SIVΔpol were inoculated (either by combination of i.v., i.m., and gene gun (g.g.) or by g.g only) into rhesus macaques (6 inoculations). Low levels of neutralizing antibodies were elicited; however, high CTL responses directed against Gag were detected and persisted for many weeks. After challenge with a lethal dose of SIV_{mac}251 (10 monkey infectious dose), animals had a rapid decline in CD4⁺ T cells and quickly succumb to infection. Although monkeys vaccinated with DNA expressing SIV239.Δpol VLP quickly reduced their viral load compared to unvaccinated animals (6 vs. 12 weeks), none of the animals were able to clear the infection. One year post-challenge, 100% (3/3) of macaques in the g.g. only group and 50% (1/2) of control macaques died from AIDS-like illness. The remaining 5 animals were free from clinical signs of disease. VLP vaccines constructed using HIV/SIV protein(s) are attenuated to a much higher degree compared to live-attenuated virus vaccines. Similar to results obtained using live-attenuated vaccines (SIVΔnef, SIVΔ3X and SIVΔ4), these VLPs corroborate that there is an inverse relationship between attenuation and efficacy with particle-based vaccines. Vaccination of monkeys with pSIV239.Δpol elicited immune responses equal to or slightly lower than the levels observed in monkeys vaccinated with

the live-attenuated strains, SIV Δ 3X and SIV Δ 4, and these vaccines did not protect the animals from live viral challenge.

The same group of researchers constructed a noninfectious HIV VLP (pNL4-3. Δ pol) based upon the SIV VLP, SIV239. Δ pol, by removing a 1.9kb DNA fragment encoding for *pol*³⁹⁹. The 5' LTR was removed and most of the 3' LTR sequences were deleted without affecting the *nef* gene. The CMV-IE promoter replaced the 5' LTR in order to efficiently initiate transcription of the vaccine insert. BALB/c mice were vaccinated (i.v, i.m., or g.g.) with 2 μ g of DNA. Antibody responses against Gag were persistent and increased with subsequent boosting, whereas Env-specific antibodies were transient, rising and falling with each successive inoculation. Total anti-Env specific antibodies were relatively low compared to live-attenuated HIV_{NL4-3} infection. However, sera from VLP vaccinated mice (1:3800) inhibited HIV-1_{NL4-3} infection of susceptible cells in an *in vitro* neutralization system (50% neutralization). Thirty-five weeks post-peak, anti-Env titers declined to undetectable levels. However, splenocytes restimulated with Env V3 loop peptides showed cytolytic activity in the vaccinated mice at this time. Overall, the immunogenicity of pNL4-3. Δ pol, as well as SIV239. Δ pol (SIV equivalent VLP), does not elicit the level of anti-HIV immunity compared to the live-attenuated parental strains.

More recently, Singh *et al.* have reported protection in macaques from disease, but not infection (3/4 monkeys), by immunization of a noninfectious SHIV VLP DNA vaccine, p Δ rtSHIV_{KU2}⁵⁹⁹. The SHIV_{KU2} provirus contains the *tat*, *rev*, *env* and *vpu* genes from HIV-1_{HXB2} and the 5' and 3' LTRs, *gag*, *PR*, *IN*, *vif*, *vpx*, *vpr* and *nef* from SIV_{mac239}. This SHIV VLP DNA vaccine contains all viral genes except RT (removal of

amino acids 364-617). Four rhesus macaques were intradermally immunized with 2 mg of pArtSHIV_{KU2} (0, 8 and 18 weeks). Neutralizing antibodies and low IFN- γ -secreting cells (ELISPOT) were detected against SHIV_{KU2}. Two weeks following the final immunization, these four animals and two unvaccinated monkeys were challenged rectally with heterologous SHIV_{89.6P} (1 ml of tissue culture medium containing 10^4 TCID₅₀ into the rectum twice, one day apart, to ensure exposure). Viral RNA titers ($>10^6$ copies/ml of plasma) and a loss of CD4⁺ T cells were detected in the unvaccinated animals two weeks following challenge. These control animals died at weeks 8 and 16 post challenge. Although all of the vaccinated animals were infected with SHIV_{89.6P}, the average of viral RNA titers were lower (average 10^4 , but ranged from 10^3 - 10^8 copies/ml of plasma), which decreased over time in 3/4 of the vaccinated monkeys. The fourth animal remained viremic and died at week 47. The immunological assays used in this study did not predict the degree of replication of the challenge virus in the vaccinated animals.

Unlike the previous VLP DNA vaccines that contained the majority of viral genes, Akahata *et. al* constructed a single plasmid that expressed Gag and Env from two different eukaryotic promoters⁹. This dual promoter expressed HIV-1 Gag from a CMV promoter in the first position, while HIV-1 Env was expressed from the Rous sarcoma virus (RSV) enhancer promoter in the second position. The Env₁₆₀ was a hybrid CCR5-tropic Env that was modified by deletion of the cleavage site (C), the fusion peptide (F) and the interspace between the two heptad repeats (I) to induce strong antibody response without weakening the cellular response. In addition, the V1 and V2 loop regions were deleted to expose core conserved determinants (gp145 Δ CFI Δ V₁V₂). The final plasmid

containing codon-optimized Gag and Env was referred to as pVLP_{gp145}. This plasmid, capable of producing virus-like particles, was compared to the same *gag* and *env* genes expressed from separate plasmids inoculated together or alone. VLPs produced from the individual plasmids did not contain Env on their surface. Mice were vaccinated intramuscularly with 50 µg of DNA (weeks 0, 3 and 6). Humoral and cellular responses were similar whether the genes were inoculated together (dual promoter plasmid or separate plasmids) or alone. Moreover, a difference in the immunogenicity of Env was not observed between the vaccines expressing Env associated with a particle or as a individual protein. Therefore, the data suggested that DNA vaccines expressing HIV-1 polyproteins (such as Gag and Env) generated immune responses similar to those elicited by virus-like particles.

A collaborative group of multiple laboratories has analyzed the immunogenicity of virus-like particles produced non-infectious, multi-gene DNA priming followed by boosting with MVA. The first group constructed multi-gene VLP DNA vaccines expressing Gag and Pol from either BH10 or HXB2 strains of HIV-1 and Env, Tat, Rev and Vpu from HIV-1_{ADA} (pJS2 and pJS7, respectively) ⁶⁰³⁻⁶⁰⁵. These vaccines were compared to DNA containing codon-optimized consensus B *gag*. Macaques were intramuscularly primed with 600 µg of DNA and boosted with two intramuscular vaccinations with 10⁸ pfu of MVA expressing HIV-1_{BH10} Gag, PR, RT and a truncated form of HIV-1_{ADA} Env. The priming of anti-Gag cellular responses was similar in monkeys vaccinated with codon-optimized or wild-type DNA. The immune responses were enhanced following the first MVA inoculation; however, the second MVA boost did enhance the anti-Env antibody titers (40-90 fold) but did not increase CD4+ or CD8+

T cell responses. Vaccination with MVA only (3 inoculations) reduced the immunogenicity 10-100 times lower for cellular responses and 2-4 times lower for antibody responses compared to the animals vaccinated with DNA/MVA. These animals were then challenged vaginally with SHIV_{89.6P}^{14, 15}. These vaccinated animals were infected with the challenge strain but were disease-free for more than 3 years. Antibodies to Env were very low or below the level of detection in monkeys vaccinated with DNA/MVA (with Env). However, monkeys vaccinated with the DNA/MVA without an Env component were not able to control viral replication as well as the monkeys vaccinated with DNA/MVA with Env¹⁴. Recently an update on this vaccination study was published⁶⁰⁶. A study using peptide pools for Gag and Env demonstrated almost complete preservation of the CD8+ T cells response but only 50% conservation of the CD4+ T cells response three years post-challenge in DNA/MVA-vaccinated animals.

The MVA component was further characterized alone in rodents by the same group⁷⁰¹ and was published simultaneously with the DNA/MVA study⁶⁰⁵. MVA/HIV 48 contains a chimeric HIV-1_{HXB-2/BH10} *gag-PR-RT* (point mutations introduced to inactivate RT) and HIV-1_{ADA} *env* (truncation of cytoplasmic tail). The *gag-PR-RT* sequences were expressed from a vaccinia virus promoter (mH5), while Env was expressed from a different vaccinia virus promoter (Psyn II). Mice were vaccinated by i.m. or i.d. with 10⁷ pfu of MVA/HIV 48 or control MVA at weeks 0 and 3. Guinea pigs were immunized at weeks 0, 1, 6 and 7 months with 10⁸ pfu of MVA/HIV 48 by i.m. or i.d., and New Zealand white rabbits were immunized (i.m.) with 2.5 X 10⁷ pfu of MVA/HIV 48 at weeks 0, 4 and 8. All three rodents elicited anti-Env antibodies but at different levels. Similar antibody titers were observed in the rabbits and guinea pigs

(10^5 - 10^6); however the antibody titers were a log lower in mice vaccinated with MVA/HIV 48. There was not a significant difference in antibody titers in animals vaccinated by i.m. or i.d. routes. In addition, the antibodies elicited by vaccinated rabbits were able to neutralize a heterologous HIV-1_{MN}, but neutralization activity did not increase after the third inoculation. Gag-specific CD8⁺ T cells were also induced by vaccination of mice with MVA/HIV 48. Unfortunately, MVA-specific CD8⁺ T cells were approximately 4-5 times higher than Gag-specific CD8⁺ T cells in these mice.

A similar vaccine approach was characterized and consisted of a multi-gene VLP DNA vaccine expressing Gag, PR, RT, Tat, Rev, Vpu and Env from the HIV-1 recombinant subtype CRF02_AG (pIC2)¹⁷³. Mutations in the PR gene of the VLP DNA were introduced to optimize the production of VLPs (pIC25, pIC48 and pIC90). Particle production was increased in these PR-modified VLP DNA vaccines compared to the VLP DNA vaccine containing wild-type PR. These vaccines were further characterized *in vitro*, but immunogenicity data was not reported.

I.I.3.d. Enhancement of immune responses in non-human primates

Persson *et al.* used a noninfectious HIV VLP that resembled live virus to elicit immune responses in rhesus macaques⁵⁰⁸. The VLP vaccine, V3_{MN}, was constructed using gene sequences from the plasmid, pMTHIV, that expresses HIV-1_{LAI} virus. The V3 loop of HIV-1_{LAI} was replaced with the V3 loop of HIV-1_{MN}, because HIV-1_{MN} is more representative of clade B HIV strains. In addition, mutations were engineered into specific sequences in the genome. A large region in the *pol* gene was deleted and

resulted in abrogation of RT and IN activity. In addition, RNA packaging signals were deleted in the RNA untranslated leader sequence and *gag* in order to prevent incorporation of genomic RNA into the budding particle. These VLPs were purified and vaccinated into rhesus macaques but only elicited low neutralizing antibodies levels (HIV-1_{MN}, 1:112-1:1473 and HIV-1_{IIIB}, <1:20-1:24, >90% inhibition).

Montefiori *et al.* focused on generating a stronger neutralizing antibody response using a slightly different VLP vaccine approach⁴⁵⁴. The noninfectious VLP vaccine, VLP_{Bx08}, is comprised of HIV-1_{LAI} Gag and PR with HIV-1_{Bx08} gp120 fused to a 28-amino acid fragment of HIV-1_{LAI} gp41. This vaccine is similar to vCP1452 except the gp120 from HIV-1_{LAI} was replaced with the gp120 from HIV-1_{Bx08}. Macaques were immunized with this VLP in one of four ways: 1) as purified VLPs, 2) expressed from DNA plasmid, 3) expressed in a canarypox (ALVAC) vector, or 4) as a combination of these strategies. Anti-Gag and anti-PR antibodies were elicited in all vaccinated monkeys regardless of how the VLP immunogen was administered. In addition, low neutralizing antibody titers were elicited in animals vaccinated with 1) VLP expressing DNA and boosted with purified VLP, 2) VLP expressing DNA and boosted with ALVAC-VLP, and 3) purified VLP alone. Induction of Gag-specific IFN- γ producing T cells were elicited by DNA expressing VLPs, whereas purified VLP immunogens or VLPs expressed by ALVAC did not.

Most previous studies using HIV VLPs have utilized gene sequences from clade B HIV-1 strains. However, in 2001, Buonaguro *et al.* constructed a VLP using gene sequences from the clade A strain of HIV-1 Gag_{pr55}⁸¹. VLPs were combined with an independent vector system that expresses Gag_{pr55}, Env_{gp120} (HIV-1_{94UG018}), Nef, and Pol

to construct the noninfectious VLP vaccine, HIV VLP_A. The Env used in this study is 90% homologous (amino acids in the V3 region) with other African HIV-1 clade-A strains. Particle formation was confirmed by western blot analysis, sucrose gradient centrifugation (1.14-1.18g/ml, 10-60%), and standard transmission electron microscopy. Several viral genes were combined to efficiently produce a noninfectious VLP with multiple targets for the immune response.

These VLPs then were used in immunogenicity studies in BALB/c mice vaccinated with multi-dose regimens of HIV VLP_A without adjuvants⁸². BALB/c mice vaccinated (i.p.) with purified VLPs (3 doses/4 inoculations) had both humoral and cell-mediated immune responses directed against Gag and Env proteins. Neutralizing antibodies prevented infection of target cells by homologous (HIV-1_{94UG018}) and heterologous (HIV-1_{IIIB}) strains in an *in vitro* neutralization assay. In addition, enhanced proliferative and CTL activity against Gag and Env were elicited.

In a different study, the levels of neutralizing antibodies appear essential for clearance of virus from infected monkeys. Notka *et al.* constructed SIV Gag_{pr56} VLPs containing the native conformation of Env or VLPs containing *env* sequences (V3 loop, CD4 binding domain, and gp41) that were incorporated into dispensable regions of *gag* (amino acids 445-464)⁴⁸⁷. The latter vaccine does not have Env incorporated into the virion surface. Both humoral and CTL responses were elicited regardless of the vaccination strategy used. In contrast to anti-Gag antibody responses, anti-Env antibody titers were not boosted by additional inoculations. After non-lethal challenge with SHIV-4 (SIV_{mac239} Gag, Pol, Vif, Nef and HIV-1_{HXBc2} Tat, Ref, Vpu, Env) (week 20, 25, 50 monkey infectious dose (MID₅₀)), all vaccinated animals became infected with the

challenge virus. Monkeys vaccinated with VLPs containing Env in its native conformation decreased viral loads (SHIV-4) more quickly than unvaccinated animals. However, clearance of virus was directly correlative with the appearance of neutralizing antibodies after challenge.

I.I.3.e. Strategies to elicit cell-mediated responses using VLPs

Various strategies have been employed to optimize the induction of CTL responses by HIV VLPs. One strategy involves the insertion of *env* gene sequences into a *gag* gene backbone, thereby directing a CTL response not only to epitopes of Gag proteins, but also Env. Schirmbeck *et al.* purified HIV VLPs (Pr55-gag/V3-3) consisting of HIV-1_{IIIB} Gag_{pr55} with sequences from the V3 loop of HIV-1_{IIIB} Env⁵⁷¹. Dispensable regions within the Gag were replaced with the V3 loop. In BALB/c mice, a single low-dose of Pr55-gag/V3-3 VLP (1µg) elicited cell-mediated responses against both Gag and Env proteins, while recombinant gp160 (10µg) alone resulted in cytotoxic responses similar to unvaccinated animals. The particles were also inoculated after sodium dodecyl sulfate (SDS)-denaturation and gel-purification was performed on Pr55-gag/V3-3. These denatured particles (1µg) were still effective for priming anti-Gag and anti-Env CTL responses and were slightly better at eliciting CTL responses than undenatured particles. However, the cytotoxicity levels were identical to SDS-denatured recombinant gp160 (1µg). Therefore, native particles or denatured monomeric proteins are capable of eliciting strong CTL responses against Gag and Env.

In a similar approach, three additional Pr55^{gag}/V3 loop VLPs efficiently elicited immune responses to HIV antigens. Wagner *et al.* determine the most favorable position to insert a sequence from the V3 loop (amino acids 296-332) within the *gag* gene sequence⁶⁶³. Also, sequences encoding for the CD4-binding domain in addition to the V3 loop (amino acids 419-444) from HIV-1_{LAI} were also inserted into this *gag* sequence. In both cases, V3 loop sequences were inserted to replace amino acids (211-241 and 436-471) in the *gag* gene. Rabbits, immunized (i.m., 3X) with purified VLPs (50µg), had strong antibody responses to Env and Gag even though low neutralizing titers were elicited. However, strong MHC I-restricted CTL activity was induced in VLP-vaccinated mice. The addition of adjuvants (incomplete Freund's adjuvant or aluminum hydroxide) to these VLP immunogens almost completely abrogated CTL activity. Interestingly, the position of V3 loop sequences within Gag did not affect the efficacy of these vaccines.

Rhesus macaques vaccinated with HIV-1_{III B} Gag_{pr55} with Env incorporated into the virion surface (strategy 1) or HIV-1_{III B} Gag_{pr55} particles replacing dispensable regions of the *gag* gene with sequences from the HIV-1_{III B} V3 loop or CD4 binding domain (strategy 2) did not elicit the levels of CTL responses observed in mice⁶⁶⁷. These VLPs were inoculated (i.m.) into rhesus macaques, without adjuvant(s). Antibodies against Gag for strategy 1 (1:8,000-1:510,000) and strategy 2 (1:4,000-1:16,000) were elicited throughout the study. Total anti-Env and neutralizing antibodies were only detected in animals vaccinated with VLPs (strategy 1) (1:2,000-1:32,000 and 1:32-1:128, >90% inhibition, respectively). Gag- and Env-specific CTL responses were similar in both groups. Four weeks after the last vaccination, macaques were challenged (i.v.) with

SHIV_{III B} (20 MID₅₀). All vaccinated animals became infected with the challenge virus in spite of anti-Env neutralizing antibody and CTL responses against Gag and Env elicited.

In another approach, Paliard *et al.* used VLPs (HIV_{III B} Gag₅₅ and HIV-1_{SF2} Env) to evaluate their ability to induce a strong CTL response without adjuvant(s) in rhesus macaques⁴⁹⁹. Monkeys, vaccinated (i.m.) with purified VLPs (200µg, 4 inoculations) elicited robust, long-lived Gag-specific cytolytic responses from isolated PBMCs and cells in the draining lymph nodes. These CTLs recognized a wide range of viral epitopes. Therefore, this type of HIV VLP has the potential to be combined with immunogens that elicit cross-reactive neutralizing antibodies to design an effective therapy against HIV in future studies.

1.1.3.f. Modifications in Env to enhance the efficacy of VLPs

Rovinski *et al.* constructed a VLP containing the full length, unprocessed HIV-1_{gp160}⁵⁵². An HIV VLP containing unprocessed gp160 (non-cleaved) was developed to compensate for the rapid loss of gp120 from the surface of particles due the weak interaction between gp120 and gp41. The unprocessed gp160 is produced by mutating the gp120/gp41 cleavage site of the chimeric HIV-1_{LAI-MN} Env_{gp160}. Subcutaneous inoculation of guinea pigs with these purified particles elicited cross-reactive neutralizing antibodies against HIV-1_{LAI/MN} and anti-Env antibodies capable of inhibiting syncytia formation and blocking Env-CD4 interactions.

Yamshchikov *et al.* generated a Gag_{pr56} SIV VLP containing different forms of SIV Env using one of two versions of Env; full length gp160 (Env_{fl}) or a truncated form

containing the complete Env sequences with a truncated cytoplasmic tail (17 amino acids) (Env_t)⁷⁰⁹. Interestingly, Env_t was transported to the cell surface and incorporated into particles more efficiently compared to Env_{fl}. Co-expression of furin resulted in more efficient cleavage of Env_{gp160} into Env_{gp120/gp41} and incorporation of Env into viral particles. Assembly of SIV VLPs with Env_{fl} was three to five times higher compared to SIV VLPs with Env_t. However, the SIV VLPs containing Env_t had more Env molecules on the surface of the particles compared to Env_{fl}. In addition, higher levels of the SIV VLPs were observed with processed (cleaved) versus non-processed Gag_{pr55} proteins similar to previous reports^{291, 478}. Therefore, the higher levels of Env_t incorporation into VLPs may be due to the more fusogenic nature of these proteins and therefore incorporation increases the host range of the particles.

Another study confirmed that truncated forms of Env increase the incorporation of Env into virions (SHIV). Yao *et al.* used purified VLPs comprised of Gag (SIV_{mac239}) and Env (HIV_{BH10} and HIV_{89.6})⁷¹⁴. This study also compared Env_{fl} or Env_t. There was a 20-50% increase in expression and incorporation of the Env_t into the virion. Cell surface expression of the Env_t was noted to be 8 fold higher than Env_{fl}. Once more, the addition of the cellular protease, furin, enhanced the cleavage of Env_{fl} into gp120/gp41.

These VLPs containing Env_{fl} and Env_t were evaluated in an immunogenicity study by the same group^{660, 661}. VLPs (Gag, SIV_{mac239} and Env, HIV_{BH10} or HIV_{89.6}), with similar levels of incorporated Env_{fl} and Env_t, elicited similar neutralizing antibody titers in mice. The VLP vaccines containing Env_t that were given as DNA induced higher levels of anti-Env specific antibodies compared to Env_{fl}. However, DNA vaccination in

combination with VLP boosting resulted in similar patterns of neutralizing antibody titers for Env_{fl} and Env_t indicating that the immunogenicity of the two forms of Env are similar. Therefore, the use of either form of Env would be useful for future development of HIV VLP vaccines.

HIV_{IIIB} gp120 covalently linked to a transmembrane domain from the Epstein Barr virus (gp120_{tmEBV}) has been shown to stabilize the envelope molecule into the virus-like particle⁴⁸⁶. SIV_{mac239} Gag_{pr56} VLPs containing gp120_{tmEBV} proteins induced significant anti-SIV antibody titers (whole virus, ELISA), T cell activity (T-cell proliferation and CTL assay) in rhesus macaques, regardless if the purified VLPs were administered alone or adsorbed to alum as an adjuvant. Vaccinated monkeys were subsequently challenged with a lethal dose of SHIV. After challenge, there was a sharp rise in virus in the blood followed by a rapid decline in viremia compared to naïve animals.

I.I.3.g. Adjuvant enhancement of VLP immunogens

A variety of adjuvants have been used to enhance the immune responses elicited by VLPs to stimulate both systemic and mucosal immune responses. Purified SIV VLPs (SIV_{mac239} Gag with Env_{fl} or Env_t) administered into the nares of BALB/c mice resulted in enhanced anti-Gag and anti-Env antibodies in the saliva and vaginal secretions but only when co-administered with the adjuvant, cholera toxin (CT)⁷¹⁵. Co-inoculation of CT with the SIV VLPs induced significantly higher anti-Env antibody responses, including higher IgA titers, compared to vaccination with SIV VLP only. Furthermore,

splenocytes and lymph nodes isolated from SIV VLP/CT vaccinated mice contained cells that expressed elevated levels of interferon-gamma (IFN- γ) and interleukin (IL)-4. Therefore, effective cell-mediated responses were elicited by mucosal inoculation.

In addition, the same group demonstrated DNA plasmids expressing a codon optimized Env in conjunction with plasmids expressing SHIV VLPs (SIV_{mac239} Gag and HIV_{89,6} Env) and purified CT (10 μ g) protein enhanced immune responses compared to Env⁷¹³. Codon optimization is described as the preferential usage of codons by different organisms. Codon optimization entails using a nucleotide gene sequence that uses codons most commonly observed in a host cell to thereby increase gene expression. For example, in human cells, tyrosine residues are most often encoded by the codon “TAC”. In HIV-1 gene sequences, tyrosines are encoded predominately by the codon “TAT”¹⁸. Converting all the codons in HIV-1 genes to the most prominent codons used by human genes increases the expression of HIV-1 gene products. One hypothesis of the enhanced protein expression of HIV-1 antigens using codon-optimized gene sequences is transcription of codon optimized genes may lead to a greater number of mRNA transcripts, which results in higher protein expression. Alternately, codon optimization may enhance protein expression by utilizing the most common and abundant tRNAs in human cells. Codon optimized genes also contain an increased GC content thereby removing AT-rich regions (intron-like inhibitory sequences) that decrease the nuclear export of mRNAs. Therefore, a DNA vaccine expressing a viral gene that has been codon-optimized or “humanized” expresses more protein in eukaryotic cells compared to the non-humanized gene. DNA expressing HIV-1 gene products can induce both humoral and cell-mediated immune responses. However, after codon optimizing, these

same viral genes elicit enhanced humoral and cell-mediated immunity^{14, 16}. DNA plasmids expressing a codon optimized Env in conjunction with plasmids expressing SHIV VLPs (SIV_{mac}239 Gag and HIV_{89.6} Env) and purified CT (10µg) protein were vaccinated into mice and rabbits. Sera collected from mice immunized with DNA expressing codon-optimized genes were able to neutralize heterologous virus (SIV_{mac}1A11) and splenocytes isolated from these animals had enhanced cellular immune responses. Mice that received CT in conjunction with DNA expressing VLPs exhibited even higher titers of anti-Env and anti-Gag antibodies, T-helper and CTL activity. After vaccination of these DNAs into New Zealand white rabbits, high levels of neutralizing antibody against SIV_{mac}251 were elicited in rabbits vaccinated with SIV_{239mac} VLPs (SIV_{mac}239 Gag and Env). Fixed SIV_{mac}239 VLPs elicited a neutralization titers against SIV_{mac}251 (1:200 at week 16, >90% inhibition). However, non-fixed SIV VLPs showed an average neutralization titer of 1:516 with one rabbit as high as 1:900.

The effectiveness of VLPs to induce anti-HIV immune responses can be improved by co-inoculating with immunostimulatory cytokines. Rhesus macaques that were primed with DNA (pVecB7) expressing the SIV VLP (SIV_{sm}B7 Gag and Env) were co-inoculated with IL-12 and GM-CSF (weeks 0, 13, and 26)⁴⁹⁰. At week 39, monkeys were inoculated with purified VLPs with or without IL-12 and subsequently challenged (intrarectally) with a lethal challenge of the viral strain SIV_{sm}E660. Five of six macaques survived the challenge, and animals that received SIV VLP DNA and cytokines (IL-12/GM-CSF as a prime and/or IL-12 as a boost) had lower viral set points than SIV VLP-DNA vaccinated or unvaccinated monkeys. In addition, control of viremia correlated

with the prevention of disease. Therefore, co-inoculation of pro-inflammatory cytokines with VLP vaccination enhances protection against viral challenge.

1.1.3.h. Human clinical trials using VLPs

There have only been a few human trials conducted using HIV VLP vaccines. The first VLP vaccine (p17/p24:Ty) has been tested in prophylactic Phase I and II trials^{419, 510}. This particle does not contain the Env protein. "Ty" is a yeast protein, which contains 25% p17 protein and 79% p24 protein (30% of the total mass of vaccine). Low levels of HIV-specific antibodies and cell mediated responses were generated in most of the volunteers after three or four immunizations in Phase I and II trials. In contrast to results found in mice immunized with this vaccine, low levels of Gag-specific CTL activity was induced in humans so far. In Phase II trials, vaccines were well tolerated and increased CD4 levels in vaccinated individuals appeared promising, but they were not statistically significant to controls. However, long term follow-up demonstrated that this vaccine had no effect on HIV disease progression in infected subjects³⁸⁹.

In 1998, Kelleher *et al.* performed a phase I clinical trial to determine the safety and efficacy of a HIV VLP composed of Gag_{p24} without envelope glycoproteins (VLP_{p24})^{38,319}. These purified VLPs were administered in combined with zidovudine (ZDV, a nucleoside reverse transcriptase inhibitor) in infected, asymptomatic HIV-1 infected patients. Patients were randomly placed into three treatment groups: 1) 200mg of ZDV (3x daily) plus inoculation (i.m.) of alum (alum hydroxide) adjuvant once per month, 2) 200mg of ZDV (3x daily) plus vaccination (i.m.) of VLP_{p24} (500µg) in alum once per

month, and 3) placebo (3x daily) plus vaccination (i.m.) of VLP_{p24} (500µg) in alum once per month. Viral load, anti-p24 antibody, and T-cell responses (CD4⁺ and CD8⁺) were similar regardless of the immunization regimen administered. Although the VLP was well-tolerated by patients (no adverse side-effects reported), VLP_{p24} in alum did not boost the anti-HIV immunity in patients on ZDV.

A VLP/canarypox virus vaccine was developed that contains Env_{gp160}, Env_{gp120}, Gag_{pr55} and Gag_{p24} and forms particles^{71, 464}. The canarypox vector virus does not replicate in human cells, but only transiently infects human cells. This transient infection results in production of a single round of HIV VLPs. This vaccine is based on a non-syncytium inducing clade B primary isolate of HIV-1 (Gag, HIV_{LAI} and Env, HIV_{MN}). Preliminary findings demonstrate that strong humoral and cellular immune responses were elicited in all recipients. However this vaccine has only been given to HIV-negative individuals (i.m.).

Few VLP vaccines have been immunized into humans. Some other Gag VLPs that are entering or are in early Phase I trials include Gag_{p24/p17} VLP plus CTL epitopes for clade A⁴⁶⁹ or B⁴⁸⁵. In the near future, multiple VLP vaccine candidates will be entering Phase I or continuing to Phase II human trials. Preliminary studies of vaccines expressing VLPs in humans have revealed positive results and support their inclusion in future trials.

I.I.4. Gag Virus-Like Particles Vaccines

Gag_{p55} is the precursor polyprotein that encodes for the main structural gene

products: MA, CA and NC (see Section I.C.1.a). In the absence of all other viral proteins and RNA, Gag_{p55} is sufficient for the assembly and formation of virus-like particles^{137, 312, 441, 666, 689}. In numerous rodent and non-human studies, Gag_{p55} VLPs have elicited strong cellular and humoral responses. Gag_{p55} VLPs can be purified similar to VLPs containing Env described in I.I.3.a. These systems include: 1) baculovirus^{142, 216, 237, 555, 635, 636, 662, 663}, 2) vaccinia virus^{246, 659, 665}, 3) Vero cells⁵⁵¹ and 4) yeast⁵⁵⁹.

Priming of the humoral response was demonstrated in rabbits and non-human primates^{139, 140, 421, 454, 662}. Gag_{p55} VLPs also induced CTL responses in non-human primates⁴⁹⁹. These cellular responses were long-lived and directed against multiple epitopes (MA, CA, p7/p6). Gag_{p55} in the form of a particle is more immunogenic than the same Gag_{p55} immunogen inoculated as a soluble protein⁴⁹⁹. Various groups have analyzed the immunogenicity of Gag_{p55} as a particle alone^{216, 559, 593}, carriers of epitopes for other viral genes^{403, 662, 666} and Gag particles with a variety of Env embedded in the particle surface^{81, 139, 140, 209, 245, 260, 349, 493, 552, 636, 659}.

Unlike its soluble counterpart, Gag_{p55} VLPs contain supplemental components or intrinsic properties that contribute to its immunogenicity. Following Gag_{p55} VLP vaccination, Gag epitopes are processed and presented on MHC I and II molecules, therefore enhancing the number and quality of T-helper and CTL responses. This broadened immunity may be due to the particulate nature of the immunogen, presence of lipids, and fusogenic antigens (in the case of Gag VLPs with Env). In addition, APC maturation and cytokine production are stimulated by VLP-associated substances derived from the expression system (i.e. viral or insect nucleic acid, immunostimulatory molecules (baculovirus gp64), cell membranes (yeast, insect), etc.). Some of these

pathogen-associated molecular patterns (PAMPs) may be recognized by the innate immune system as “danger signals”. Recognition of PAMPs results in upregulation of costimulatory molecules (B7.1 and B7.2) and cytokines (IL-12 and IL-18), which leads to an enhanced and broadened cellular (Th1) and humoral immune response.

I.I.5. Application of VLP vaccines

Two primary strategies for the production of particle-based lentiviral vaccines have been reviewed: 1) replication-competent virus that integrates into the host genome, persistently infecting the host (live-attenuated virus) or 2) viral proteins inoculated as *ex vivo*, purified particles or expressed from DNA *in vivo* to form non-infectious, non-replicating, non-pathogenic particles (virus-like particles). Live-attenuated virus is effective at eliciting protective immune responses against HIV in non-human primate models. Live-attenuated virus elicits a robust, broad CTL response, as well as high levels of cross-reactive neutralizing antibodies. These vaccines persistently express viral antigens and therefore usually require fewer boosts. In addition, live-attenuated viruses contain multiple viral antigens in native conformation(s) that can infect professional APCs. However, live-attenuated virus vaccines for HIV have some significant drawbacks that need to be overcome before they can be widely used in patients. The most significant safety issue is the potential of live-attenuated HIV to revert to a pathogenic form after inoculation, as well as the possibility to recombine with wild-type virus in an infected host. Another problem with live-attenuated HIV vaccines is the

degree of attenuation is either insufficient and therefore the vaccine causes disease or these vaccines are overly attenuated as to not elicit protective immune responses.

An alternative approach to live-attenuated virus vaccines for HIV is the use of virus-like particle vaccines. These VLPs mimic the virion particle without the concerns for safety of live-attenuated viruses. Some of the advantageous characteristics of VLP vaccines include the 1) induction of humoral/cell-mediated and systemic/mucosal immune responses, 2) multi-gene composition, 3) expression of native conformation(s) of Env, 4) presentation of viral peptides on MHC I and II molecules, and 5) infection of APCs. Nonetheless, additional parameters need to be addressed in order for lentiviral VLPs to elicit protective immune responses such as the dose of inoculum and the number of immunizations. In addition, future research needs to focus on overcoming the inverse relationship between the degree of attenuation and efficacy of particle-based vaccines. The ability of the vaccine to protect the host from infection decreases as the number of genes deleted or rendered nonfunctional to attenuate the virus increases. The use of effective adjuvants co-administered with VLPs may assist in these efforts. There are multiple particle-based HIV vaccines in pre-clinical and clinical trials and the future of these vaccines appear promising for the induction of anti-HIV immunity.

I.J. Mucosal Vaccination and Immune Response

Many pathogens gain access to the host through surfaces such as the respiratory, gastrointestinal, vaginal and rectal mucosa. The mucosal immune system is comprised of an integrated network of lymphoid cells that interacts with innate host factors to preserve

the integrity of the host defense. HIV can infect Langerhans cells located in the pluristratified epithelial layer of the vagina or dendritic cells in the rectal cavity and transverse the intact epithelial barrier¹⁰⁰. These dendritic cells have high expression of DC-SIGN which promotes the internalization of the virus^{211, 277, 360, 481, 618, 685}. Consequently, HIV infects a substantial number of CD4+ T cells found in these tissues. Eventually, this localized infection rapidly leads to widespread infection of the host. In contrast to the viral load in the blood, treatment with antiviral drugs does not lower the viral titer in the rectal mucosa¹⁴⁷. In addition, Langerhans cells in the epithelium and lamina propria in the vagina and rectal mucosa can act as reservoirs of viral infection^{46, 147, 515}.

Protection against mucosal pathogens is conferred by a combination of antibodies (IgG and sIgA) and CTLs^{35, 37, 67, 350, 368}. IgG and sIgA block HIV infection of susceptible cells *in vitro*³⁴⁷. These anti-HIV antibodies can prevent viral transcytosis across a mucosal monolayer^{10, 54}, neutralize virus infectivity¹⁴⁶, and mediate antibody-dependent cellular cytotoxicity⁵³. Additionally, containment of viral replication by lysis of infected cells is achieved primarily by CTLs⁴²⁴. The number of antigen-specific cytotoxic CD8+ T cells in the lamina propria of the gut is directly proportional to the level of protection after mucosal viral challenge^{35, 425, 466}. Therefore, augmentation of the immune response at the mucosa by mucosally-administered vaccines may induce high titer anti-HIV immunity and is more likely to prevent viral infection and the establishment of viral reservoirs compared to vaccines inoculated parenterally.

HIV-1, similar to many other pathogens, is primarily transmitted at the mucosal surface. Yet, the majority of experimental vaccines against HIV-1 have been inoculated

parenterally. Although an efficient systemic immune response develops, immunity at the mucosa after parenteral inoculation is negligible. In contrast, mucosal immunization can induce immune responses at both local and distal mucosal sites as well as systemic immunity^{100, 128, 372, 376}. HIV vaccines have been inoculated at multiple mucosal sites in the form of 1) protein/peptides^{36, 374, 445, 615}, 2) bacteria^{363, 531, 584, 585, 651}, 3) DNA^{15, 22, 26, 306, 402, 564, 669}, 4) virus^{37, 77, 117, 165, 177, 185, 620} and 5) VLPs^{331, 373, 390, 451, 647, 715}. In contrast to HIV, successful vaccination of animals and humans with VLPs has been achieved with viral pathogens such as papillomavirus⁵⁹⁰, Norwalk virus²³⁹, hepatitis E virus^{385, 482} and Sindbis virus⁶⁴⁷.

I.K. Cytosine Phosphate Guanosine Oligodeoxynucleotides

Synthetic oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine motifs (CpG ODNs) can be used as an immune adjuvant. Unmethylated CpG motifs are prevalent in bacterial but not in mammalian genomic DNA^{186, 219, 708}. The relevant sequence motif consists of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines³⁵³. This motif is found approximately 20 times more often in bacteria compared to mammalian DNA due to the utilization and methylation patterns of CpG dinucleotides in prokaryotes versus eukaryotes^{87, 532}. CpG ODNs activate host defense mechanisms against a variety of pathogens (viral, allergy, fungal, and tumor immunogens), which lead to innate and acquired immune responses in a variety of fish, birds and mammals⁵²⁸.

The recognition of CpG ODNs is through Toll-like receptor (TLR) -9, which instigates alterations in cellular redox balance and the induction of cell signaling pathways in both rodents and primates^{264, 625}. These pathways include the mitogen activated protein kinases (MAPKs) and NF- κ B, which lead to direct upregulation of cytokine/chemokine gene expression^{7, 244}. Cells that do not express TLR-9 or TLR-9 knockout mice are unresponsive to CpG ODNs^{7, 17, 244, 625}. TLR-9 is a member of a receptor family that mediates responses to PAMPs expressed by a variety of infectious pathogens⁴³⁸.

In humans, cells that express TLR-9 include plasmacytoid dendritic cells, macrophages and B cells. Activation of these cells leads to production of pro-inflammatory (IL-1, IL-6, IL-18, TNF- α) or Th1 cytokines (IFN- γ and IL-12), interferons (IFN- α,β) and chemokines^{249, 339, 353}. CpG ODNs also induce the polyclonal activation of IgM secreting cells (including memory B cells)^{43, 353, 717} and the maturation of professional APCs²⁴⁰. In contrast, mammalian DNA and oligodeoxynucleotides lacking unmethylated CpG motifs do not induce cytokine secretion or APC maturation^{240, 339}. Natural killer (NK) cells are activated secondarily, secreting IFN- γ and gaining cytolytic activity. In addition, B cells become more sensitive to activation through their antigen receptor. The expression of costimulatory molecules is enhanced on APCs, which improves their ability to activate T-cell responses (IgG2a).

Co-vaccination of CpGs with a variety of immunogens has improved the immunity to pathogens in various animal challenge models. Vaccination of CpG ODNs with influenza^{262, 451}, measles³⁴⁵, hepatitis B^{69, 428}, tetanus¹⁶⁹ and HIV^{714, 715} immunogens leads to increased antibody responses, CTL activity and IFN- γ secretion.

Enhancement of the immune response by CpG motifs has been demonstrated with DNA^{336, 458, 545, 566, 645} and protein vaccines^{66, 69, 126, 169, 450}. Ongoing human clinical trials indicate that CpG ODNs, administered as adjuvants, are safe, well-tolerated and have augmented the vaccine-elicited immune responses^{222, 351, 352}.

CpG ODNs have also been used as mucosal adjuvants. Immune responses were enhanced by co-immunization of CpG ODNs with influenza⁴⁵⁰, Hepatitis B^{274, 429} and HIV immunogens^{714, 715}. The immune response induced by co-inoculation with CpG ODNs was comparable to vaccination with other adjuvants such as cholera toxin (CT) and RANTES⁷¹⁵.

I.L. Blocks in HIV-1 Replication in Rodents

HIV-1 productively infects and causes disease in humans and chimpanzees but not in rodents. There are multiple species-specific restrictions in HIV-1 replication in rodents that are derived from the dependency of the virus on host cellular functions for replication. In particular, HIV-1 does not replicate in murine cell lines in vitro, nor does HIV-1 infect laboratory mice (inbred or outbred)⁴⁶¹.

There are a number of reasons for the failure of HIV-1 to replicate in murine cells. First, there is a block in viral entry. HIV-1 Env does not readily bind to murine CD4 (mCD4), mCCR5 nor mCXCR4^{39, 330, 364, 406}. Expression of hCD4 with either hCXCR4 or hCCR5 on murine cells permits HIV-1 to enter these cells, however little or no productive replication occurs^{47, 207, 417}. Similar results are seen in transgenic mice (hCD4

plus hCCR5 or hCXCR4) in vivo following infection with HIV-1^{73, 568}; cells are infected but viremia is not detected.

In addition to the block in virus entry in murine cells, there are several post-entry blocks in rodent cell lines. Tat trans-activation is inefficient in murine cells, which results in low levels of viral protein expression^{12, 206, 259, 480}. The host gene responsible for this block is human cyclin T1 (hCycT1), which is a Tat-interacting protein and a component of the pTEFb transcription factor complex. Murine CycT1 (mCycT1) differs from its human homolog by several amino acids, but the presence of a tyrosine rather than a cysteine at position 261 is responsible for its interaction with Tat^{49, 207, 359}. Expression of hCycT1 in murine cells restores Tat function and along with hCD4 and appropriate coreceptor, allows HIV-1 replication to proceed from viral entry through proviral gene expression^{47, 417}.

More recently, research has been focused on elucidating the mechanisms responsible for the late blocks in HIV-1 replication. Controversy has surfaced regarding a possible defect in the function of Rev in rodent cells. In some studies, only a small reduction was noted in the abundance of unspliced viral mRNAs, which suggested that the defect was minor^{322, 417}. Whereas, other studies revealed a more profound defect in Rev function in several rodent cell types^{47, 641}. Although the discrepancy of Rev function remains unclear, it may be due to differences in cell types rather than species. The reduction in unspliced viral mRNA in certain rodent cells may be due to oversplicing, reduced stability of unspliced transcripts or some other mechanism yet to be determined. Regardless, the relative amount of unspliced viral mRNAs found in the cytoplasm did not correlate with the magnitude of infectious virus production from multiple cell types⁴⁷.

Therefore, if a defect in Rev exists in certain rodent cell types, it is unlikely to contribute significantly to the prevention or decreased production of infectious virus ^{47, 417}. However, the contribution of a defective Rev in the failure of HIV to replicate in rodent cells is still under investigation.

HIV-1, similar to other complex retroviruses, uses the Rev/Response Element (RRE) system in conjunction with the Crm-1 dependent pathway for nuclear export of unspliced and singly-spliced viral mRNAs. Analogous to the nuclear export pathway mediated by Rev, the constitutive transport element from the Mason-Pfizer monkey virus (MPMV CTE) can also facilitate the nuclear export of unspliced and singly-spliced viral mRNAs ^{68, 731}. Simple retroviruses and other RNA and DNA viruses contain CTE or CTE-like sequences that interact with cellular proteins to facilitate nuclear export of viral mRNAs ^{258, 692}. These cellular proteins include two nucleocytoplasmic shuttle proteins: TAP (NXF1) (mRNA export factor) ^{238, 309} and the adenosine 5'-triphosphate-dependent RNA helicase A ⁶²⁷ and may include others ⁵⁰⁵. Addition of one copy of the MPMV CTE effectively replaces the Rev/RRE system of HIV and SIV, but with a lower efficiency ^{68, 540, 731}. The addition of multiple copies of the MPMV CTE overcomes this restriction and is actually more efficient than the Rev/RRE system ⁶⁹³. These two systems can functionally replace one another presumably due to the use of common cellular factors (such as Sam68) ^{533, 534}. In HIV-1, RRE must be present in the mRNA, but the location of the RRE within the exon is not important for its function in the Rev/RRE system ⁸⁶. Unlike the Rev/RRE system, the close association of the CTE in relation to the poly A sequence is critical for its function in the genomic or the subgenomic context ^{467, 539}.

One of the major obstacles in the development of a rodent model for HIV-1 infection is a potent block at the stage of virus assembly. This type of replication block is relatively rare for most mammalian retroviruses, which can undergo virion assembly and release from a variety of cell types. For example, HIV-1 VLPs can assemble and release from yeast spheroplasts that have had their cell wall removed⁵⁵⁹. The specific mechanism of the block in assembly has yet to be elucidated. Intravesicular Gag aggregates are observed by electron microscopy in HIV-infected murine cells⁴¹⁷. This data suggests that Gag may be trapped within intracellular compartments and therefore may be responsible for the block in assembly.

Controversy also surrounds the non-infectious nature of the particles derived from rodent cells. Some studies have reported particles derived from rodent or human cells were equally infectious based on TCID₅₀:p24 ratios^{322, 417}. In contrast, another study indicated virions produced in rat or mouse cells were largely non-infectious⁴⁷.

The underlying mechanism responsible for the block in virion assembly and release in murine cells has yet to be clarified. One theory proposed includes the mistargeting of Gag, which may result in the accumulation of Gag in intracellular vesicles⁴¹⁶. There are many cellular proteins that play a role in the assembly process of HIV in human cells including Tsg101 (vacuolar protein sorting)⁶⁵³ and HP68 (post-translational events in immature capsid assembly)⁷³⁰. However, inhibiting the function of these proteins in human cells resulted in a block in viral egress, but the phenotypes were dissimilar to the phenotypes seen in murine cells. These results suggest the involvement of other unknown host factors (cellular protein or a lipid component of the plasma membrane). Further research will need to focus on the mechanism of the block in

virion assembly and release in rodent cells. Most likely, this block in the replication cycle is due to a missing host factor rather than some other component of the cell. Since HIV-1 VLPs have been reported to assemble and bud from *Saccharomyces* spheroplasts, this model may be manipulated in the future to identify the missing factor(s) essential for HIV-1 egress.

II. Chapter 2: Material and Methods

II.A. DNA Plasmids

The lineage of all the plasmids is outlined in Table 2. Digestion (Section II.A.1), ligation (Section II.A.2) bacterial transformation and amplification (Section II.A.3), extraction and purification from agarose gels (Section II.A.4), PCR (Section II.A.5), and SDM-PCR (Section II.A.6) and sequencing of plasmid DNA (Section II.A.7) were all performed as described. The plasmids were stored at 4°C (short term) or -20°C (long term).

II.A.1. Digestion of DNA with Restriction Enzymes

Plasmid DNA was digested in a 1.5 ml centrifuge tube in the following reaction: 500 ng (vector plasmids) or 1 µg (insert plasmid) of plasmid DNA, 2 µl of enzyme buffer (final concentration, 1X) (Table 2) and 1 µl of each restriction enzyme in a final volume of 20 µl. The samples are placed at 37°C for 1-4 h (1 h for diagnostics, 2-4 h for molecular cloning). The digested DNA was analyzed by agarose gel electrophoresis. Digested DNA was mixed with 1X blue sucrose loading dye (Invitrogen Life Technologies, Carlsbad, CA, USA) and loaded onto a 1% agarose gel (1% agarose in TAE buffer (40mM Tris acetate, 2mM Na₂ EDTA (pH 8.0) in distilled water) along with a 1kb ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). The samples were electrophoresed (70-80V) for 1-2 h. Digested DNA was analyzed for fragment size using a Transiluminator ultraviolet light (Fisher Scientific, Pittsburgh, PA, USA) and a Photo-Documentation Camera (Fisher Scientific, Pittsburgh, PA, USA).

II.A.2. Ligation of DNA Fragments

Plasmid DNA was digested and purified as described in Sections II.A.1 and II.A.4. DNA fragments were ligated using the following reaction: vector and insert fragments (vector to insert ratios: 1:1, 1:2 and 1:4) were added to 1 μ l of 10X T4 DNA ligation buffer, 1 μ l (1 U/ μ l) of T4 DNA ligase (Invitrogen Life Technologies, Carlsbad, CA, USA) in a total volume of 10 μ l of distilled water. The samples were incubated at 16° C for 4 h followed by transformation into *E. coli* XL-gold cells (see Section II.A.3).

II.A.3. DNA Amplification and Purification from Bacteria

Competent cells

Bacterial transformation was used to introduce plasmid DNA into competent bacterial cells. The bacterial cells used for transformation, chemically competent XL10-Gold cells, are derived from XL10-Gold ultracompetent cells (Invitrogen Life Technologies, Carlsbad, CA, USA). XL10-Gold ultracompetent cells (50 μ l) were thawed on ice for 10 min. The cells were added to 200 ml of Luria Broth (LB) and incubated for 16 h at 37°C (225 rpm). 2.5% of the overnight culture was added to 200 ml of fresh LB broth and incubated at 37°C with shaking until the O.D. reading at 550 nm is 0.3 using a BioMate 3 spectrophotometer (Thermo Spectronics, Rochester, NY, USA). LB broth alone was used as the blank. The bacterial culture was placed into four 50 ml conical tubes (U.S.A. Scientific, Ocala, FL, USA) and incubated on ice for 15 min. The cells were pelleted by centrifugation (3,000 rpm for 5 min) at 4°C. The supernatants

were decanted, and each pellet was resuspended in 16 ml of Transformation Buffer #1 (99.3M RbCl, 48.5M MnCl₂, 1M KOAc, 10.2M CaCl₂, 15% glycerol, pH 5.8). The samples were mixed thoroughly, and the cells were pelleted by centrifugation (3,000 rpm for 5 min) at 4°C. The supernatants were decanted, and each pellet was resuspended in 4 ml of Transformation Buffer #2 (0.5M MOPS (pH, 6.8), 19.9M RbCl, 150M CaCl₂, 30% glycerol) and pooled together. The sample was mixed thoroughly and placed into 1.5 ml centrifuge tubes (200µl each). The cells were flash-frozen in an ethanol-dry ice bath for 1 min and stored at -80°C.

Bacterial Transformation and DNA Amplification

Plasmid DNA (50-200 ng) was mixed with 20 µl (~1.0 x 10⁶ cells) of chemically competent XL10-Gold cells in a 1.5 ml microcentrifuge tube (Fischer Scientific, Middletown, VA, USA). The mixture was incubated on ice for 30 min, and incubated in a 42^o C water bath for 45 sec. The mixture was returned to ice for an additional 5 min. 80 µl of sterile LB (EZmix: enzymatic casein digest 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, and inert binder 0.6 g/L) (Sigma, St. Louis, MO, USA) was added to the mixture. The mixture was incubated at 37^o C with shaking at 200 rpm for 1 h. The entire mixture was then transferred onto a pre-warmed, room-temperature, antibiotic agar plate (ampicillin or kanamycin) (Sigma, St. Louis, MO, USA) (50 µg/µl). Sterile glass beads (10-12 beads per plate) (Fischer Scientific, Middletown, VA, USA) were added onto the plate and used to spread the mixture evenly by shaking the plate back and forth several times. The plate was inverted and incubated in a 37°C incubator for 18 h. A bacterial colony was picked from the plate and used to inoculate 5 ml of sterile LB containing 0.01

$\mu\text{g}/\mu\text{l}$ antibiotic (kanamycin or ampicillin). The culture was incubated at 37°C with shaking at 200-250 rpm for 8 h. 1 ml of the culture was then added to 200 ml of LB containing $0.01 \mu\text{g}/\mu\text{l}$ antibiotic. The culture was then incubated at 37°C with shaking at 200-250 rpm for 15-18 h.

Bacterial Purification

Bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C in a 250 ml polyclear centrifuge tube (Nalgene, Rochester, NY, USA) using a RC5C centrifuge (Sorvall Instruments, Newtown, CT, USA). The supernatant was decanted and the bacterial pellet was resuspended in 4 ml of Qiagen Buffer P1 (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, and $100 \mu\text{l}/\text{ml}$ RNase A) (Quiagen Inc, Valencia, CA, USA). The bacterial cells were transferred to a 50 ml tube and 4 ml of Qiagen Buffer P2 (200 mM NaOH and 1% SDS) was added to lyse the cells. The solution was mixed by gently inverting the tube 4-6 times and incubated at room temperature for no more than 5 min. The lysis process was neutralized by adding 6 ml of pre-chilled Qiagen Buffer P3 (3.0 M potassium acetate, pH 5.5). The solution was gently mixed by inverting the tube 4-6 times and incubated on ice for 20 min. The samples were centrifuged at 20,000 rpm for 30 min at 4°C . The supernatant containing plasmid DNA was transferred into a new tube. The sample was centrifuged at 20,000 rpm for an additional 15 min at 4°C . A Qiagen-tip 100 column was equilibrated by applying 4 ml of Qiagen Buffer QBT (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100). The column was allowed to drain by gravity flow. The supernatant containing the plasmid DNA was then applied to the column and allowed to drain by gravity flow. The column

was washed two times with 10 ml of Qiagen Wash Buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol). The plasmid DNA was eluted with 5 ml of Qiagen Buffer QF (1.25 M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol) into a new tube. Plasmid DNA was precipitated by adding 3.5 ml of room temperature isopropanol to the 5 ml of eluted DNA. The sample was immediately mixed and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was discarded. The plasmid DNA pellet was washed with 2 ml of 70% ethanol and centrifuged at 15,000 rpm for an additional 10 min. The supernatant was discarded and the pellet was air-dried overnight at room temperature or 1 h at 37°C. The plasmid DNA pellet was resuspended in distilled water. A sample of each DNA was diluted 1:200 (5 µl of DNA in 995 µl of distilled water), the concentration of DNA was determined using a BioMate 3 spectrophotometer (Thermo Spectronics, Rochester, NY, USA). The optical density (wavelength, 260/280 nm) of the diluted DNA sample was measured by the spectrophotometer. The optical density of non-DNA containing distilled water was subtracted from the recorded optical density of the diluted DNA to calculate the final optical density. The concentration of DNA was determined by the following: DNA concentration (C, µg/µl) = $[A_{260} / 0.020 (\mu\text{g/ml})^{-1}] \times (1 \text{ ml}/1000\mu\text{l})$.

II.A.4. Extraction and Purification of DNA from Standard Agarose Gels

Restriction enzyme-digested DNA was extracted and purified from agarose gels. After separation of restriction enzyme-digested DNA fragments by electrophoresis, the extracted and purified DNA was used for the construction of the various molecular clones (Table 2). The restriction enzyme-digested DNA was removed from the agarose gel (1%

agarose in TAE, 40 mM Tris Acetate, 2 mM Na₂EDTA (pH 8.0) in distilled water) (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) by physical separation with a razor blade and placed in a 1.5 µl microcentrifuge tube and weighed. DNA fragments were separated from the agarose using a QIAquick gel extraction kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's recommended protocol. Briefly, three volumes of Buffer QG (proprietary compound mixture, 50-100% guanidinium thiocyanate, pH indicator) were added to one volume of agarose gel. For example, 300 µl of Buffer QG was added to 100 mg of gel. The microcentrifuge tube was incubated at 50°C for 10 min or until the agarose was completely dissolved. The tube was vortexed every two to three minutes to help dissolve the gel. One volume of isopropanol (Sigma, St. Louis, MO, USA) was then added to one volume of gel. The dissolved mixture was overlaid into a QIAquick ion-exchange spin column was placed in a collection tube and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded, and the QIAquick spin column was placed back in the same collection tube. The sample was washed with 750 µl of Buffer PE (proprietary compound mixture, 44.4% ethanol) and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded and the QIAquick spin column was placed back in the same collection tube. The QIAquick spin column was centrifuged for an additional 1 min at 10,000 rpm and the flow-through was discarded. The QIAquick spin column was placed in a new 1.5 µl microcentrifuge tube. The DNA was eluted by adding 30 µl of distilled water to the center of the QIAquick membrane (25°C for 1 min). The sample was then centrifuged for 1 min at 10,000 rpm, the spin column was then discarded, and the eluted DNA was stored at 4°C.

Table 2. DNA plasmids.

Plasmid[†]	Description
pHIV-1 _{BH10}	HIV-1 BH10 provirus (pBC12 vector)
pHIV-1 _{ADA}	HIV-1 ADA provirus (pBC12 vector)
pHIV-1 _{89.6}	HIV-1 89.6 provirus (pBC12 vector)
pVLP _{BH10/wt}	HIV-1 BH10 VLP: <i>gag</i> , PR, RT, <i>env</i> , <i>vpu</i> , <i>tat</i> , <i>rev</i> -no safety mutations
pVLP _{BH10}	HIV-1 BH10 VLP: <i>gag</i> , PR, RT, <i>env</i> , <i>vpu</i> , <i>tat</i> , <i>rev</i> + safety mutations
pVLP _{ADA}	pVLP _{BH10} + HIV-1 ADA <i>env</i> , <i>vpu</i> , <i>tat</i> , <i>rev</i> + safety mutations
pVLP _{89.6}	pVLP _{ADA} + HIV-1 89.6 <i>env</i>
pVLP _{R2}	pVLP _{ADA} + HIV-1 R2 <i>env</i>
pVLP _{JR-FL}	pVLP _{ADA} + HIV-1 JR-FL <i>env</i>
pVLP _{coADA}	pVLP _{ADA} + codon optimized HIV-1 ADA <i>env</i>
pVLP _{coR2}	pVLP _{ADA} + codon optimized HIV-1 R2 <i>env</i>
pVLP _{ADA/2xCTE}	pVLP _{ADA} + 2 copies of MPMV-CTE + 200bp nonsense sequence
pVLP _{ADA/ΔRT}	pVLP _{ADA} without RT
pVLP _{SHIV/ADA}	pVLP _{ADA} + SIV mac239 <i>gag</i> , PR, RT-no safety mutations
pVLP _{SHIV/ADA/ΔRT}	pVLP _{SHIV/ADA} without RT
pLTR-SEAP	HIV-1 5' LTR + secreted alkaline phosphatase (pBC12 vector)
pCD4	4 extracellular domains of human CD4 (soluble)
pcoADA _{gp120}	codon optimized HIV-1 ADA Env gp120
pcoR2 _{gp120}	codon optimized HIV-1 R2 Env gp120
pGag	codon optimized HIV-1 NL4-3 Gag
pGag _{ΔMyr}	codon optimized HIV-1 NL4-3 Gag without myristylation site
pGag-Pol	codon optimized HIV-1 NL4-3 Gag-Pol

[†] pTR600 is the base vector unless otherwise noted.

II.A.5. Polymerase Chain Reaction (PCR)

In vitro polymerase chain reaction was used to amplify gene products. Plasmid DNA (50 ng) with the sequence of interest was added to a microcentrifuge tube with 2 synthetic oligonucleotide primers (1 µg/µl), ~30 nucleotides in length. Each reaction mixture (Table 3) was placed in a Robocycler[®] Gradient 96 thermal cycler (Stratagene, La Jolla, CA, USA). A DNA fragment was amplified using a three step reaction cycle (Table 3). The amplified product was cloned into pCR2.1-TOPO plasmid vector using the TOPO TA Cloning Kit[®] (Invitrogen Life Sciences, Carlsbad, CA, USA). Each amplified fragment (2 µl) was incubated in a 1.5 ml centrifuge tube in the following conditions: 1 µl of salt solution (1.2 M NaCl, 0.06 M MgCl₂), 1 µl of pCR2.1-TOPO vector (10 ng/µl plasmid are in: 50% glycerol, 50 mM Tris-HCl, pH 7.4 (25°C), 1 mM EDTA, 1 mM DTT, 0.1% Triton-X 100, 100 µg/ml BSA, phenol red), and 2 µl of distilled water for 5-20 minutes at 25°C. 2 µl of the ligation reaction was then incubated in 50 µl One Shot[®] TOP10 Competent Cells (*E. coli*, F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74* *recA1* *araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*) (Invitrogen Life Sciences, Carlsbad, CA, USA) on ice for 5-30 min. The samples were heat-shocked for 30 sec at 42°C and then returned to 4°C for 2-3 min. S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) (250 µl) was added to the competent cell mixture. The

Table 3. Polymerase chain reaction cycling parameters.

Cycles[†]	Temperature (°C)	Time
1 (hot start)	95	1 minute
30	95	1 minute
	55	1 minute
	72	2 minutes

[†]Each reaction contains 200 ng of plasmid DNA, 5 µl of 10X reaction buffer (100mM Tris-HCl, pH 8.3 (at 42°C) 500mM KCl, 25mM MgCl₂, 0.01% gelatin), 50mM of dNTP (12.5mM dATP, 12.5mM dCTP, 12.5mM dGTP, 12.5 mM dTTP, neutralized at pH 8.0 in water), 1 µg of each oligonucleotide primer, 1 U of Taq DNA polymerase (1 U/µl) (Stratagene Cloning System, La Jolla, CA, USA) and distilled water added to final volume of 50µl.

Table 4. Site-directed mutagenesis polymerase chain reaction cycling parameters.

Cycles[†]	Temperature (°C)	Time
1 (hot start)	95	1 minute
18	95	50 seconds
	60	50 seconds
	68	2 minutes x kb of the plasmid
1 (clean-up)	70	10 minutes

[†]Each reaction contains 50 ng of plasmid DNA, 5 µl of 10X reaction buffer (100mM Tris-HCl, pH 8.3 (at 42°C) 500mM KCl, 25mM MgCl₂, 0.01% gelatin), 50mM of dNTP (12.5mM dATP, 12.5mM dCTP, 12.5mM dGTP, 12.5 mM dTTP, neutralized at pH 8.0 in water), 125 ng of each oligonucleotide primer, 2.5 U of Pfu Turbo Taq DNA polymerase (2.5 U/µl) (Stratagene Cloning System, La Jolla, CA, USA) and distilled water added to final volume of 50µl.

mixture was incubated at 37°C for 1 h with shaking (200 rpm). The samples were spread using glass beads onto pre-warmed antibiotic selective agar plates (ampicillin or kanamycin), and incubated for 16-18 h in at 37°C.

II.A.6. Site-Directed Mutagenesis Polymerase Chain Reaction

In vitro site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) introduced point mutations in DNA sequences using synthetic complementary oligonucleotide primers (~30 nucleotides in length) containing the desired mutations (Table 4). The reaction mixture was placed in a Robocycler® Gradient 96 thermal cycler (Stratagene, La Jolla, CA, USA). The plasmids were amplified using the cycling parameters described in Table 4. Then, each reaction mixture was digested with *Dpn I* (2 Units) and incubated at 37°C for 2-4 hours. XL-10 Gold competent cells were transformed with a sample of *Dpn I*-digested DNA following the same protocol as described in Section II.A.3.

II.A.7. DNA Sequencing

DNA plasmids that were modified by site-directed mutagenesis were often sequenced to verify the mutation using the dye terminator cycle sequencing method^{261, 284}. A Model 373 DNA Sequencing System (Applied Biosystems, Foster City, CA, USA) at the East Carolina University Genomics Core Facility or the 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA) at the University of Pittsburgh Genomics and Proteonomics Core Laboratories and the BigDye v3.0 chemistry from ABI

was used for the sequencing reactions. A reaction mixture of double-stranded plasmid DNA (750 ng), specific oligonucleotide primers (10 μ M each), Bigdye (containing excess amounts of the four deoxynucleotide triphosphates (dNTP), fluorescence tagged dideoxynucleotide triphosphate (ddNTP) terminators, AmpliTaq DNA polymerase, magnesium chloride, and distilled water. Each ddNTP terminator was tagged with a different fluorescent molecule. The reaction mixtures were electrophoresed in a high resolution polyacrylamide gel under denaturing conditions, so the DNA remains single-stranded. During electrophoresis, a laser excites the fluorescence tags and a charged-couple device (CCD) camera detects the signal and sends it to a computer for interpretation. The data were analyzed by DNA Sequencing Analysis software or Chromas (Technelysium, Tewantin, Australia). DNA sequencing results were recorded as a text file and as a 4-color histogram.

II.B. Cell Culture

II.B.1. Transfections

Cell Viability

The efficient expression of each plasmid was determined by transient transfection of plasmid DNA into mammalian cells. Cell lines were plated at a concentration of approximately 5.0×10^5 cells per well in 6-well plates (35 mm²) (Becton Dickinson, Piscataway, NJ, USA). Cell viability was determined by trypan blue exclusion cell counting. Cells were detached from the flask using 3ml of 1X trypsin (0.05% trypsin,

0.4% EDTA·4Na) (Gibco, Grand Island, NY, USA) for 3-5 min at RT. The cells were triturated with 7 ml of complete Dulbecco's Modified Eagle Medium (DMEM) (cDMEM) [DMEM supplemented to contain 10% heat-inactivated fetal bovine serum (FBS) (1 h at 56° C) (Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), and 0.4 mg/L gentamicin (Gibco, Grand Island, NY, USA)] and placed in a 50 ml conical tube. After thorough mixing, 10 μ l of the cell suspension was incubated for 5 min with 10 μ l of 0.4% Trypan Blue solution (Gibco, Grand Island, NY, USA) and half of the solution was added to each side of a hemacytometer (VWR, Bridgeport, NJ, USA). Within the hemacytometer, the cells within the four outer quadrants were counted using a light microscope (100X power) (Fryer Company, Inc, Huntley, IL, USA). Only the non-blue cells were counted and the average of the four quadrants was recorded. The number of viable cells was determined with the following equation: # viable cells (cells/ml) = (average number of cells in 4 quadrants) X dilution factor for size of quadrant (i.e. 10,000) X dilution factor for addition of trypan blue. Each sample was performed in duplicate to accurately determine the cell number. Cell counts within 10% of each other were considered accurate. The total number of cells within the flask was determined by the following equation: Total cells in flask = cells/ml X # ml in flask.

DNA Transfection

After the cell count was determined, the cells were seeded (5×10^5 cells/well, 6-well plate) allowed to acclimate for 24 hours at 37° C plus 5% CO₂. The medium was then removed by aspiration, and cells were washed with 1 ml of DMEM (Gibco, Grand

Island, NY, USA). The plates were rocked back and forth to thoroughly wash the cells. The wash medium was aspirated, and the cells were replenished with 1 ml of DMEM. The cells were then transfected with the DNA listed in Table 2. DNA plasmid (2 µg) was incubated with Lipofectamine™ reagent (2 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA) in DMEM at room temperature for 45 min. Each transfection mixture was added to a well of the 6-well plate and incubated at 37° C plus 5% CO₂. After 18-24 h, the transfection mixture was replaced with 2 ml of cDMEM. Cells were incubated for an additional 48 h at 37° C in a humidified 5% CO₂ incubator. Forty-eight hours post-transfection, the supernatant were harvested and placed into 1.5 ml centrifuge tube. The supernatant was clarified by centrifugation (1 min for 10,000 rpm) and transferred to a new 1.5 ml centrifuge tube and stored at -80° C. After the supernatants were removed, the cells were washed with 1 ml of phosphate buffered saline (PBS) (137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 2.7 mM KCl, in distilled water) (Gibco, Grand Island, NY, USA). The plates were rocked back and forth to thoroughly wash the cells. The wash solution was aspirated, and the cells were incubated with either 300 µl of distilled water or 1 % Triton-X 100 (Sigma, St. Louis, MO, USA) until the cells completely detached from the plate. The samples were incubated at -80° C for 5-10 min and then thawed at room temperature for 5-10 min. Freeze/thawing was repeated two times. The cell lysate samples were centrifuged (1 min at 10,000 rpm) and the supernatants were transferred to a new 1.5 ml centrifuge tube and stored at -80°C.

II.B.2. Human Embryonic Kidney (293T)

The human (*Homo sapiens*) embryonic kidney (HEK or 293) cell line is adherent. 293 cells are transformed with adenovirus 5 DNA (39768) and require Biosafety Level 2 facilities. 293T is a highly transfectable derivative of the 293 cell line (American Type Culture Collection, (ATCC), CRL-1573) into which the temperature sensitive gene for simian virus 40 (SV40) T-antigen was inserted^{234, 586}. The cells were maintained according to the American Type Culture Collection (ATCC) recommended growth conditions in cDMEM at 37° C in a humidified 5% CO₂ incubator.

II.B.3. COS-7

COS-7 cells are an adherent, kidney fibroblast cell line derived from the African green monkey (*Cercopithecus aethiops*) (ATCC, CRL-1651)²²³. These cells contain SV-40 viral DNA sequences, which require Biosafety Level 2 facilities. This line was derived from the CV-1 cell line (ATCC, CCL-70) by transformation with an origin defective mutant of SV40, which encodes for wild-type T antigen. The cells were maintained according to the ATCC recommended growth conditions in cDMEM at 37° C in a humidified 5% CO₂ incubator.

II.B.4. NIH/3T3

The NIH/3T3 cell line was used for *in vitro* expression experiments for the

plasmids described in Table 2. NIH/3T3 is an adherent, embryo fibroblast cell line derived from the NIH/Swiss mouse (*Mus musculus*) (ATCC, CRL-1658)²⁹⁵. NIH/3T3, a continuous cell line of highly contact-inhibited cells was established from NIH Swiss mouse embryo cultures similar to the original randomly bred 3T3 (ATCC, CCL-92) and the inbred BALB/c 3T3 (ATCC, CCL-163) [Jainchill, 1969 #28]. The established NIH/3T3 line was subjected to more than 5 serial cycles of sub-cloning to develop a sub-clone with morphologic characteristics optimized for transformation assays. The cells were maintained according to the ATCC recommended growth conditions in cDMEM at 37° C in a humidified 5% CO₂ incubator.

II.B.5. BALB/c 10ME HD A.5R.1 (BC10ME)

BC10ME is an adherent, embryonic fibroblast cell line derived from the BALB/c mouse (*Mus musculus*)⁵⁰⁶. This cell line is derived from BALB/c CL.7 (ATCC TIB-80) by transformation with methylcholanthrene epoxide. BC10ME_{Gag} and BC10ME_{Pol} were produced by stable transfection of BC10ME with HIV-1_{NL4-3} Gag and Pol DNA sequences, respectively²⁸³. The cells were maintained according to the ATCC recommended growth conditions in cDMEM at 37° C in a humidified 5% CO₂ incubator.

II.B.6. TZM bl

TZM bl is an adherent, cervical epithelial cell line derived from the HeLa-CD4/CCR5 (JC53) human (*Homo sapiens*) cell line^{517, 677}, which is susceptible to

infection with both R5 and X4 HIV-1 isolates. It is maximally sensitive to infection by including DEAE-dextran in the medium. Using an HIV-based vector, genes that encode the *Escherichia coli* β -galactosidase (β -gal) and firefly luciferase (LUC) coding sequences were introduced into the JC53 cell line. These cells contain papovavirus, which require Biosafety Level 2 facilities. The cells were maintained according to the ATCC recommended growth conditions in cDMEM at 37° C in a humidified 5% CO₂ incubator.

II.C. Protein Expression

II.C.1. Tat Reporter Assay

Tat protein activity was determined by an *in vitro* reporter assay⁴⁰. 293T cells (5.0 X 10⁵/well, 6 well-plate) were transiently transfected with 1 μ g of each VLP vaccine and 1 μ g of the reporter plasmid, pLTR-SEAP as described in Section II.B.1. pLTR-SEAP expresses the secreted alkaline phosphatase (SEAP) gene under the control of the HIV-1 promoter located in the HIV-1 5' LTR. After 18 h, media was replenished with 1.5 ml of cDMEM and the cells were incubated an additional 48 h. Cell supernatants were collected and incubated at 65°C for 5 min to inactivate endogenous phosphates. The samples were analyzed for SEAP activity using a colorimetric assay. 2X SEAP buffer (100 μ l) (2 M diethanolamine, 1 mM MgCl₂, 20 mM L-homoarginine) was incubated with 100 μ l of each sample in a 96 well plate for 10 min at 37°C. 20 μ l of paranitrophenol (PNP) (120 mM) (Sigma, St Louis, MO, USA) was added and incubated

for 30 min at 37° C in the dark. The colorimetric change was measured as the optical density (O.D. at 405 nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The non-specific SEAP activity was detected in cells transfected with pTR600 vector and was subtracted from each test sample.

II.C.2. Western Blot

Mammalian cells were transfected with DNA and samples were collected as described in Section II.B.1. For Western blotting, cell lysate (3.3%, 10 µl) or supernatant (1.5%, 30 µl) was diluted (1:2) in sodium dodecyl sulfate (SDS) Laemmli sample buffer (1M Tris-Cl pH 6.8, 20% SDS, 3.3 % Glycerol, 0.006 M bromophenol blue and 0.05 M beta-mercaptoethanol) (Bio-Rad, Hercules, CA, USA). The mixture was briefly vortexed and then boiled for 4 min. The sample mixtures and a pre-stained molecular weight ladder were loaded onto a 5-10% SDS-polyacrylamide gel (Stacking Gel: 30% acrylamide:bis, 10% SDS, 10% ammonium persulfate, 1% TEMED, 0.5 M Tris-HCl pH 6.8 or Resolving Gel: 1.5 M Tris-HCl, pH 8.8). The proteins were separated by electrophoresis at 100 V for 20 min followed by 175 V for 1 h in running buffer (25 M Tris base, 192 M glycine, 0.1% SDS, pH 8.3). The resolved proteins were transferred from the SDS-PAGE to a Immobilon™ nitrocellulose membrane (Millipore, Bedford, MA, USA) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol, pH 8.3) using the transfer apparatus (Mini Trans-Blot, Bio-Rad, Hercules, CA, USA) for 1 h at 200 mA. The nitrocellulose membrane was removed and placed in blocking solution (5% dry non-fat milk, 0.05% Tween 20, PBS) overnight at 4° C with gentle rocking. The blocking solution was replaced with polyclonal antiserum (HIV Ig) (NIH ARRRP,

Germantown, MD, USA) (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk) and gently rocked for 1 h at room temperature. The nitrocellulose membrane was then washed 3 times for 10 min each in 25 ml of PBS supplemented with 0.05% Tween 20 (PBS-T) on a shaker at 25° C. The membrane was then incubated with anti-human IgG conjugated to horseradish peroxidase (HRP) (Bio-Rad Laboratories, Hercules, CA) (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk) on a shaker for 1 h. The membrane was then washed 3 times for 10 min each in 25 ml of PBS-T on a shaker at 25° C. The membrane was washed for 20 min in 25 ml of PBS on a shaker at 25° C. The membrane was then incubated with 1 ml of stable peroxide solution and 1 ml of lumino/enhancer solution (Amersham, Buckinghamshire, UK) and manually rocked for 3 to 4 min. The membrane was then briefly dried, wrapped in cellophane, and exposed to CL-X Posure™ film (Pierce, Rockford, IL, USA) for 10 sec to 5 min.

II.C.3. BCA Protein Assay

The concentration of protein was determined using a Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). A protein standard (2 mg/ml of bovine serum albumin (BSA) in a solution of 0.9% saline and 0.05% sodium azide) was used to produce a standard curve (Figure 4). Standard protein concentrations or test samples (150 µl) and 150 µl of the working reagent [52% Micro BCA Reagent A (sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2N NaOH), 48 % Micro BCA Reagent B (4% bicinchoninic acid in water), 2% Micro BCA Reagent C (4% cupric sulfate, pentahydrate in water)] were pipetted into the wells of a 96 well plate (Corning,

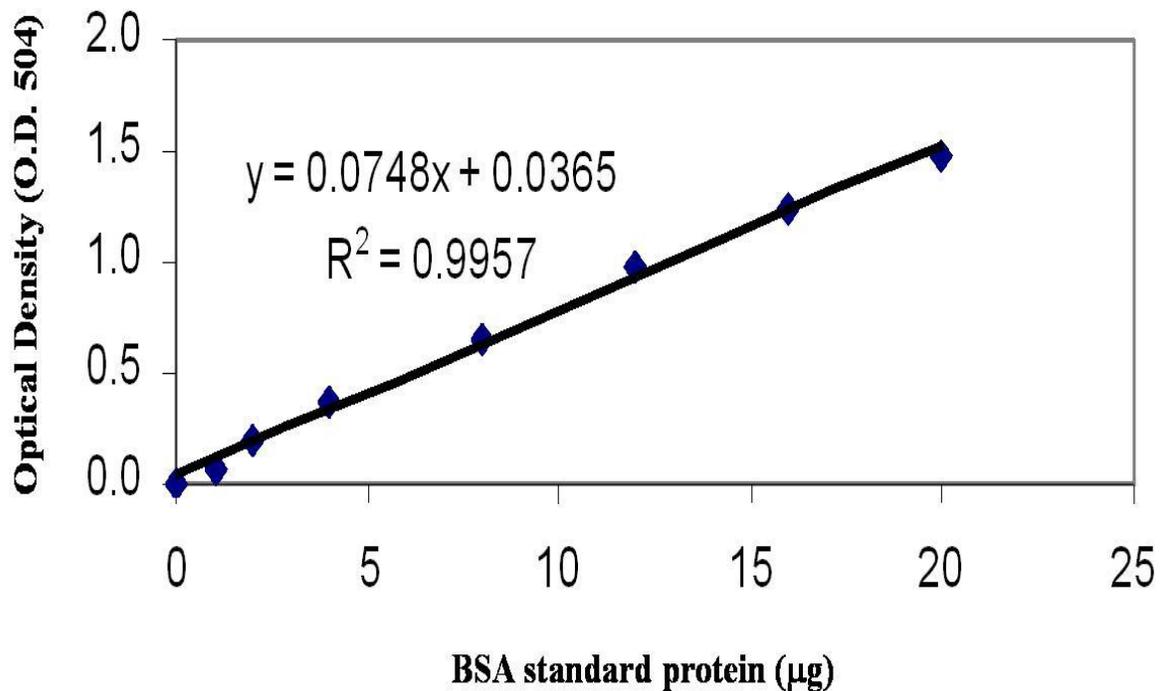


Figure 4. BSA standard curve for protein quantitation.

Total protein content was determined for the purified VLP inoculum used for the vaccination studies. The concentration of protein was determined using a Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used as the protein (BSA) to produce a standard curve. Standard protein concentrations or test samples were analyzed for a colorimetric change measured as the optical density (O.D. at 540nm) by a spectrophotometer. Tests were performed in duplicate at two different dilutions, whereas the standard samples were analyzed in triplicate. The concentration of protein in the test samples was extrapolated using the standard curve.

Corning, NY, USA). The plates were covered and placed on a shaker for 1 h at 37°C. The samples were analyzed for a colorimetric change measured as the optical density (O.D. at 540nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Tests were performed in duplicate at two different dilutions, whereas the standard samples were analyzed in triplicate. The O.D. of each sample was compared to the standard curve, and the concentration of the protein was extrapolated from the standard curve (Figure 4).

II.C.4. Silver Stain

The purity of purified proteins or particles were verified by silver staining²¹⁵ using a ProteoSilver Stain Kit (Sigma, St. Louis, MO, USA) and the manufacturer's recommended protocol. Samples were prepared and electrophoresed as described in Sections II.B.1 and II.C.1. After electrophoresis of the proteins by SDS-PAGE, the gel was placed in a clean tray with 100 ml of Fixing Solution (50% ethanol, 10% acetic acid in ultrapure water) for 20 min. The Fixing Solution was decanted, and the gel was washed with 100 ml of Ethanol Solution (30% ethanol in ultrapure water) for 10 min. The Ethanol Solution was removed, and the gel was washed twice (10 min) with 200 ml of ultrapure water (Sigma, St. Louis, MO, USA). After removal of the water, the gel was incubated with 100 ml of Sensitization Solution (1% ProteoSilver Sensitizer in ultrapure water). The Sensitization Solution was removed, and the gel was washed twice (10 min) with 200 ml of ultrapure water. The water was decanted and 100 ml of Silver Equilibration Solution was added to the gel for 10 min. After the Silver Equilibration Solution was removed, the gel was washed for 60-90 sec 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) was added to the tray for 3-7 min until the desired staining intensity was observed. 5 ml of the ProteoSilver Stop Solution was added to the Developer Solution to stop the reaction (5 min).

II.D. CD4 Binding Assay

A CD4 binding assay was used to determine whether envelopes (purified gp120 or on the surface of a particle) expressed from DNA bound the primary receptor, hCD4. DNA expressing VLPs or gp120 (5 µg) was transiently transfected into COS cells (1 X 10⁶ cells per T75 flask (U.S.A. Scientific, Ocala, FL, USA) as described in Section II.B.1. Briefly, DNA plasmid (5 µg) was incubated with 5% lipofectamine (30 µl in 600 µl of DMEM) for 45 min. The cells were overlaid with the DNA-lipofectamine mixture in DMEM (5 ml) for 16 h. The cells were replenished with fresh cDMEM (9 ml) and incubated for 48 h. Supernatants were collected and clarified by centrifugation (2,000 rpm for 5 min). Then supernatant (9 ml) was incubated with 16 µg of hCD4 (NIH ARRRP, Germantown, MD, USA) (4 h at 25°C) and pelleted through 20% sucrose (w/v in PBS) (Invitrogen, Carlsbad, CA, USA) (2ml) for 2 h at 100,000 X g (24,000 rpm) (SW40Ti rotor) (Kendro Laboratory Products, Newtown, CT, USA). The pellets were analyzed CD4 by Western blot (see Section II.C.2). hCD4 was detected using mouse anti-CD4 (1:3,000)⁵⁹. The primary antibody was detected using goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:7,000) (Southern Biotechnology

Associates, Birmingham, AL, USA). Detection of the protein-antibody complexes was enhanced by chemiluminescence and visualized by autoradiography (see Section II.C.2).

II.E. Particle Purification

Cells were plated at a concentration of approximately $5-7 \times 10^6$ cells per T75 flasks (U.S.A. Scientific, Ocala, FL, USA). The cells were allowed to acclimate for 24 h at 37° C plus 5% CO₂. The wash medium was aspirated, and the cells were replenished with 5 ml of DMEM. The cells were then transfected with the DNA listed in Table 2. DNA plasmid (5 µg) was incubated with Lipofectamine™ reagent (30 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA) in DMEM (600 µl) at room temperature for 45 min. Each transfection mixture was added to a T75 flask and incubated at 37° C plus 5% CO₂. After 18-24 h, the transfection mixture was replaced with 10 ml of cDMEM. Cells were incubated for an additional 48 h at 37° C in a humidified 5% CO₂ incubator. Post-transfection (48 h), the supernatants were clarified by centrifugation (3,000 rpm for 10 min) at 4°C and the supernatant (10 ml) was transferred to a 12 ml polyclear ultracentrifuge tubes (Kendro Laboratory Products, Ashville, NC, USA) and underlaid with 1 ml of 20% glycerol (w/v in PBS) (Sigma, St. Louis, MO, USA). Each sample was balanced with an Explorer Pro analytical scale (Ohaus Inc., Pine Brook, NJ, USA) and placed in a pre-chilled TH641 rotor (Kendro Laboratory Products, Ashville, NC, USA). The rotor was placed into a Sorvall Discovery 905E ultracentrifuge (4°C) (Kendro Laboratory Products, Newtown, CT, USA) and the samples were ultracentrifuged (100,000 x g (24,000 rpm) for 2 h) at 4°C. The supernatants were decanted and the

pellets were rinsed one time with sterile PBS. The pellets were then resuspended in 500 μ l sterile PBS and used for: 1) *in vivo* immunization or 2) sucrose density gradient ultracentrifugation.

Pellets used for immunization were analyzed for total protein by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) as described in 2.3.3. In addition, the level of non-HIV protein contamination (from the media/cell debris) in each inoculum was visualized by a silver-stained SDS-PAGE using the ProteoSilver Silver Stain Kit (Sigma, St. Louis, MO, USA) as described in Section II.C.4. The specificity of each inoculum was analyzed in a separate immunoblot that was probed with HIV-Ig as described in Section II.C.2.

Pellets analyzed for particle formation were resuspended in PBS (500 μ L) and overlaid onto a 20-60% sucrose gradient (Figure 5). The sucrose gradient was made by adding 1 ml of each fraction (11 steps, 4% increments) starting at the bottom of the tube with 60% sucrose (w/v in PBS) and overlaying subsequent fractions (60%-20% of sucrose). The glycerol pellet was added to the top of the gradient, and the tubes were balanced using an analytical scale. The tubes were loaded onto the TH641 rotor and ultracentrifuged for 17 h at 100,000 x g (24,000 rpm) at 4°C. Eleven fractions (20-60%, 1 ml each) were collected (top to bottom) from the gradient and placed into 2.0 ml centrifuge tubes (Brinkmann/Eppendorf, Westbury, NY USA). The proteins were precipitated with 1 ml of 20% trichloroacetic acid (TCA) (30 min at 4°C) and centrifuged for 20 min at 4°C. The supernatants were decanted; the protein was resuspended with Laemmli sample buffer plus 2 μ l of 5N NaOH and subjected to SDS-PAGE and immunoblotting as described in Section II.C.2.

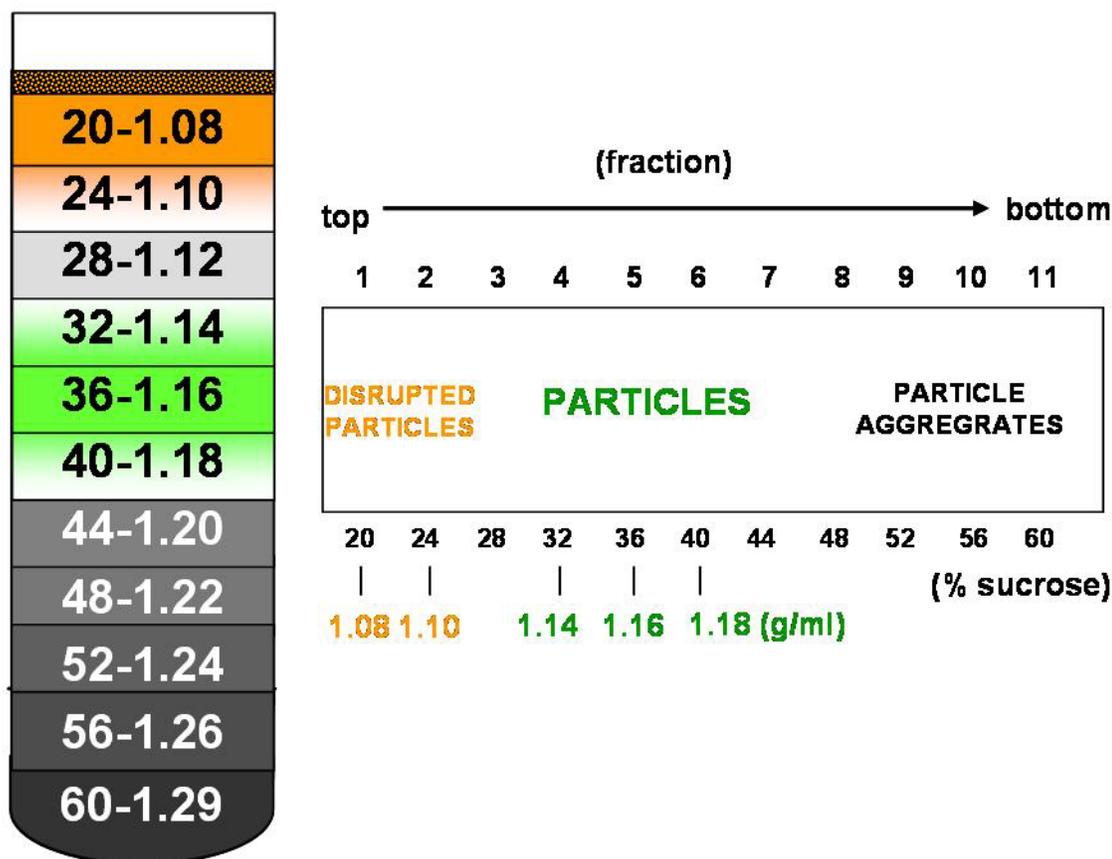


Figure 5. Schematic of virus-like particle production by sucrose density gradient ultracentrifugation.

Supernatant from COS cells transiently transfected with VLP DNA was concentrated through a 20% glycerol cushion, resuspended and then subjected to 20-60% (w/v) sucrose density gradient ultracentrifugation (16 h, 100000 x g). Fractions were collected from top to bottom, precipitated with equal volumes of 20% TCA and proteins were detected by western blot analysis. Percent of sucrose fraction is located at the bottom of each lane: lane 1, 20%; lane 2, 24%; lane 3, 28%; lane 4, 32%; lane 5, 36%; lane 6, 40%; lane 7, 44%; lane 8, 48%; lane 9, 52%, lane 10, 56%, lane 11, 60%. All samples were electrophoresed through a 5-10% polyacrylamide/SDS gel and transferred to a PVDF membrane. Proteins were immunoblotted with HIV-1 Ig and mouse anti-human IgG and visualized by enhanced chemiluminescence. Fractions are indicated where disrupted particles, intact particles and particle aggregates sediment in the 20-60% gradient. Percentage of sucrose and densities (g/ml) are specified for the different fractions on the tube and western blot illustrations.

II.F. BALB/c Mice

II.F.1. Husbandry

Five to 7 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN, USA) were used for vaccination studies. The mice were housed (5 mice per cage, free access to food and water, cages cleaned weekly) in compliance with the U.S. Department of Agriculture (USDA) regulations. Mice were monitored daily for weight loss, behavior, and adverse reaction. Mice were anesthetized based on their weight with xylazine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (20 mg/ml) and ketamine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (100 mg/ml) administered subcutaneously (50 mg ketamine and 5 mg xylazine per kilogram of body weight, average 20 g) in the abdomen prior to immunization and blood collection. Mice were sacrificed by CO₂ (100%) inhalation.

II.F.2. Intramuscular Inoculation

Female BALB/c (5-7 weeks old) mice were intramuscularly immunized with DNA (5 µg), purified protein (10 µg) or purified VLPs (40 µg total protein) alone or co-inoculated with phosphorothioate CpG oligodeoxynucleotides (CpG ODN, 10 µg each) via the hind muscle. All intramuscular inoculations were performed in a total of 50 µl of sterile PBS. Each CpG ODN (ODN-1: 5'-TCCATGACGTTTCCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3') was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG

ODNs were resuspended in sterile distilled water (2 µg/µl) and stored at -80°C. Mice were administered VLP vaccines (DNA or purified particles) in sterile PBS +/- CpG ODN into the hind leg of each mouse (50 µl total volume) using a 1 ml syringe (Becton Dickinson & Co., Franklin Lakes, NJ, USA).

II.F.3. Intranasal Inoculation

Female BALB/c mice (5–7 weeks old) were immunized with purified protein (10 µg) or purified VLPs (40 µg total protein) alone or co-inoculated with CpG ODN (10 µg each) via the nares. All intranasal inoculations were performed in a total of 40 µl of sterile PBS. Each CpG ODN (ODN-1: 5'-TCCATGACGTTTCCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3') was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile distilled water (2 µg/µl) and stored at -80°C. Mice were administered VLPs in sterile PBS +/- CpG ODN into the nares of each mouse (40 µl total volume) using a 100 µl Pipetman.

II.F.4. Blood Collection, Mucosal Washings and Organ Harvesting

Blood samples were collected by retro-orbital plexus puncture using a heparinized capillary tube (Drummond Scientific Company, Broomall, PA, USA) and a 1.5 ml centrifuge tube on anesthetized mice and incubated (4° C for 16 h) to allow coagulation of the red blood cells. Serum was separated from the red blood cells by centrifugation (5,000 rpm for 10 min). Collected serum was stored in at -80° C.

Three mucosal surfaces were washed to collect antibody elicited by vaccination: vagina, lungs, and intestines. Vaginal lavages were collected on anesthetized mice by repeated rinsing of the vagina with 200 μ l of sterile PBS using a 200 μ l Pipetman. Following euthanasia (described in Section II.F.1), lungs were excised using surgical scissors (Fischer Scientific, Middletown, VA, USA) and dissected into small pieces using a sterile scalpel (Fischer Scientific, Middletown, VA, USA) and placed in sterile PBS (200 μ l). The lung matter was centrifuged (12,000 rpm for 5 min). A section of the intestines (1.27 cm) was aseptically removed using surgical scissors and placed in sterile PBS (200 μ l). The collected fecal samples were disrupted using a sterile pipette tip, vortexed (15 sec), and centrifuged (12,000 rpm for 5 min). The protein concentration of the fecal supernatants was adjusted to a final concentration of 1 mg/ml with sterile PBS using the Micro BCA Protein Assay Kit as described in Section II.C.3. All samples were stored in at -80°C.

Spleens were removed by making an incision from the rib cage to the tail using a sterile scalpel and tweezers. The spleen was removed using surgical scissors and placed in sterile PBS (10 ml volume in a 15 ml conical tube) (U.S.A. Scientific, Ocala, FL, USA). The spleen was then placed on a wire mesh (Fischer Scientific, Middletown, VA, USA) over a 60 x 15 mm dish (U.S.A. Scientific, Ocala, FL, USA). The splenocytes were manipulated into a single cell suspension using the rubber stopper end of a 5 ml syringe plunger (Becton Dickinson & Co., Franklin Lakes, NJ, USA). The cell suspension was transferred to a new 15 ml conical tube and incubated on ice (5 min) to allow larger debris to settle to the bottom of the tube and the cell suspension was then transferred to a new 15 ml conical tube. The cells were centrifuged at 1500 rpm (5 min at

4°C) and the supernatants were discarded. The cells were resuspended in 1 ml of ACK solution (0.155M ammonium chloride, 0.1M Na₂EDTA, 0.01M potassium bicarbonate in distilled water) and incubated on ice (3 min). PBS (10 ml at 4°C) was added to the tube, mixed gently, and centrifuged at 1500 rpm (5 min at 4°C). The supernatants were discarded, and the cells were washed with PBS and centrifuged again. The cells were then resuspended in 2-5 ml of complete Roswell Park Memorial Institute (cRPMI) media (RPMI supplemented to contain 10% heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA, USA), 4 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.4 mg/L, 0.01% penicillin-streptomycin (Gibco, Grand Island, NY, USA)) and used in assays described in Section II.H.3 and II.H.4.

II.G. New Zealand White Rabbits

II.G.1. Husbandry

Five to 7 week old New Zealand white rabbits (Harlan Sprague Dawley, Indianapolis, IN, USA) were used for vaccination studies. The rabbits were housed (1 rabbit per cage, free access to food and water, cages cleaned weekly) in compliance with the U.S. Department of Agriculture (USDA) regulations. Rabbits were monitored daily for weight loss, behavior, and adverse reaction. Rabbits were anesthetized based on their weight with xylazine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (20 mg/ml) and ketamine (Phoenix Pharmaceutical, Inc, St. Joseph, MO, USA) (100 mg/ml)

administered subcutaneously (40 mg ketamine and 5 mg xylazine per kilogram of body weight, average 2 kg) in the hind leg prior to immunization.

II.G.2. Intramuscular Inoculation

Five to 7 week old New Zealand white rabbits were used for the vaccination study. Each rabbit was anesthetized (see Section II.G.1) and inoculated with 200 µg of VLP DNA in 500 µl of sterile PBS intramuscularly with a 20-gauge (Becton Dickinson, Piscataway, NJ, USA) needle. The inoculum was divided in half and inoculated equally in both quadriceps muscles.

II.G.3. Blood Collection and PBMC Harvesting

Blood samples were collected at week 20 by ear venipuncture using a 5 ml syringe (Becton Dickinson, Piscataway, NJ, USA) and allowed to coagulate. The whole blood was transferred to a 15 ml conical tube (U.S.A. Scientific, Ocala, FL, USA), centrifuged at 3,000 rpm (5 min at 4°C). The sera was removed from the red blood cells and stored at -80°C.

Peripheral blood mononuclear cells were harvested from vaccinated rabbits. Ficoll Paque (Pharmacia Biotech, Wikstroms, Sweden) (3ml) was added to a 15 ml conical tube (USA Scientific, Ocala, FL, USA), and blood samples (4ml) were layered on top. Without mixing, the samples were centrifuged (1,000 rpm for 30 min) at 18-20°C. The plasma (upper layer) was removed, leaving the lymphocyte layer (buffy coat) at the interface. The lymphocyte layer was then removed and added to a new 15 ml conical

tube containing salt solution (0.1% anhydrous D-glucose, 5×10^{-5} M $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 9.8×10^{-4} M $\text{MgCl}_2 \times 6 \text{H}_2\text{O}$, 5.4×10^{-3} M KCl, 0.145M TRIS, 0.14M NaCl) (6 ml). The cells were resuspended and centrifuged (3,000 rpm for 10 min) 18-20°C. The supernatant was removed and the cells were washed again with salt solution and centrifuged. The supernatant was removed and the cells were resuspended in cRPMI. Cell viability was determined by trypan blue exclusion cell counting (see Section II.B.1).

II.H. Immunological Assays

II.H.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Sera and mucosal samples were individually collected and tested for antibody responses to VLP antigens by ELISA. Each well of a 96-well plate (Corning, Corning, NY, USA) was coated with culture supernatant from cells transfected with DNA expressing $\text{Env}_{\text{gp120}}$ from genes codon optimized for mammalian cells ($\text{pcoADA}_{\text{gp120}}$ or $\text{pcoR2}_{\text{gp120}}$) (50 ng) or purified Gag_{p55} protein (100 ng) (NIH ARRRP, Germantown, MD, USA) in PBS (total volume, 100 μl) at 4°C. Plates were blocked (25°C for 2 h) with PBS (200 μl) containing Tween 20 (0.05%) and non-fat dry milk (5%). The blocking buffer was removed and 100 μl of serially diluted samples (sera or mucosal washings) was added to each well (25° C for 2 h). Following thorough washing (3X) in PBS-Tween 20 (0.05%), samples were incubated (25° C for 1 h) with 100 μl of biotinylated goat anti-mouse IgG (1:5,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was

removed, and the wells were washed three times. Streptavidin-HRP (1:7,000) (Southern Biotechnology Associates, Inc., Birmingham, AL) was diluted in 100 μ l of PBS-Tween 20 (0.05%) and incubated (25°C for 1 h). 100 μ l of TMB substrate (1 TMB tablet per 10 ml of phosphate-citrate pH 5.0 buffer; 2 μ l 30% H₂O₂) (Sigma, St Louis, MO, USA) was added to each well (25°C for 1 h), and the colorimetric change was measured as the O.D. by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Results were recorded as the arithmetic mean plus the standard deviation (S.D.) after the value of naïve sera was subtracted from the test samples.

In order to measure the levels of HIV-1 Env-specific IgG subtypes, a modification to the above protocol included the use of biotinylated goat anti-mouse IgG1 or IgG2a antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) to detect Env-antibody complexes (1:5,000) in place of the biotinylated goat anti-mouse IgG.

II.H.2. Neutralization Assay

In vitro virus neutralization by antisera from vaccinated mice was determined by a luciferase reporter infection assay. TZM bl cells (NIH ARRRP, Germantown, MD, USA) were plated (1 X 10⁵ cells/well) in a 96 well plate (Corning, Corning, NY, USA). Twenty hours later, purified HIV-1_{ADA} (1300 TCID₅₀) or HIV_{MN} (750 TCID₅₀) (NIH ARRRP, Germantown, MD, USA) was incubated with heat-inactivated antisera (1:10) (56°C for 1 h) from naïve or vaccinated mice (1 h at 37°C, 5% CO₂). After 1-2 h, the virus-sera mixture was added to the 96 well plate containing TZM bl cells in a total volume of 100 μ l of DEAE dextran (Sigma, St. Louis, MO, USA) (20 μ g/ml) in DMEM. The virus-sera mixture was aspirated and the wells were washed twice with cDMEM

(200 μ l). The cells were overlaid with cDMEM (200 μ l) and incubated at 37°C (5% CO₂) for 48 h. Lysates were collected (see Section II.C.2) and assayed for the presence of luciferase using the Promega Luciferase Assay System (Promega, Madison, WI, USA). Luciferase reagents were gently thawed at 25°C for 30 min. The cell lysates were briefly vortexed and then centrifuged at 2,000 rpm for 1 min. The lysates were transferred to a new 1.5 ml microcentrifuge tube and placed on ice. The luciferase substrate (2 mg/ml) was added to a new 1.5 ml microcentrifuge tube. Next, 20 μ l of cell lysates were added to the substrate microcentrifuge tube and mixed by trituration. The luciferase reaction was measured using a Fentomaster FB12 luminometer (Zylux Corporation, Oak Ridge, TN, USA), and the value was recorded as relative light units per second (RLU/sec). The parameters of the instrument were set for a 2 sec delay and a 10 sec reading time. Inhibition of virus was assessed by the additional reduction in infectivity beyond the background of mock-vaccinated and pre-bleed antisera. The neutralizing capacity of antisera was measured by comparing the reduction in luciferase activity per sample to sera from age-matched, mock-vaccinated mice. Each mouse sample was analyzed in duplicate assays on the same day and averaged. Results are given as: % Inhibition of Infection = [(Experimental – Age-Matched, Mock-Vaccinated) \div Virus Alone] X 100. The arithmetic mean \pm the S.D. was determined for each group after the appropriate background values were subtracted from the test samples.

II.H.3. Enzyme-Linked Immuno-Spot (ELISPOT) Assay

Splenocytes were isolated as described in Section II.F.4. Cell viability was

determined by trypan blue exclusion staining (Gibco BRL, Grand Island, NY, USA) (see 2.2.1). The number of anti-Gag_{p24} or anti-Env_{gp160} murine INF- γ (mINF- γ) secreting splenocytes was determined by an enzyme-linked immunospot (ELISPOT) assay kit (R & D Systems, Minneapolis, MN, USA). Pre-coated anti-mINF- γ plates were incubated (25°C for 2 h) with RPMI supplemented with 10% FBS (200 ml) and then were incubated with splenocytes (1 X 10⁶/well) isolated from vaccinated mice. Splenocytes were stimulated for 48 h with peptides representing the HIV-1 Gag_{p24} immunodominant epitope (HIV-1 consensus B Gag: GHQAAMQMLKETINE and AMQMLKETINEEAAE)⁴²³, 2 μ g of purified, soluble Gag_{p55} or Env_{gp160} (North America consensus sequence: KSIHIGPGRAFYTTG and B-subtype isolate: KSIPMGPGKAFYATG) (NIH ARRRP, Germantown MD, USA). Additional wells of splenocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng) (Sigma, St. Louis, MO, USA) and ionomycin (500 ng) (Sigma, St. Louis, MO, USA) or were mock stimulated. All wells received 10 U/ml of mIL-2 (NIH ARRRP, Germantown, MD, USA). Plates were washed with PBS-Tween (3X) and were incubated (25°C for 2 h) with biotinylated anti-mINF- γ (2 μ g/ml, mAb XMG1.2) (R & D Systems, Minneapolis, MN, USA) at 4°C for 16 h. The plates were washed and incubated (25°C for 2 h) with streptavidin conjugated to alkaline phosphatase (R & D Systems, Minneapolis, MN, USA). Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1 h) with stable 5-Bromo-4-chloro-3-indolyl phosphate dipotassium nitrotetrazolium blue chloride (BCIP/NBT) chromagen (R & D Systems, Minneapolis, MN, USA). The plates were rinsed with distilled water and air dried (25°C for 1-2 h). Spots were counted by an ImmunoSpot ELISPOT reader (Cellular

Technology Ltd., Cleveland, OH, USA). The number of spots per 1×10^6 splenocytes was recorded after subtracting the background values (naïve mice stimulated with Gag peptides or proteins and unstimulated splenocytes from test samples).

Results are given as: Number of spots/ 1×10^6 splenocytes = [(Experimental_{protein/peptide stimulated} - Experimental_{mock stimulated}) - (Naïve_{protein/peptide stimulated} - Naïve_{mock stimulated})]. The arithmetic mean \pm the S.D. was determined for each group after the appropriate background values were subtracted from the test samples.

II.H.4. Cytotoxicity Assay

The same Gag_{p24} peptides and Gag_{p55} protein (NIH ARRRP, Germantown, MD, USA) (see Section II.H.3) were used in a cytotoxicity assay (CytoTox 96 Non Radioactive Cytotoxicity Assay kit) (Promega, Madison, WI, USA). A mixture of peptides (1 μ g of each peptide) and protein (2 μ g) was incubated with the splenocytes (1×10^7 cells/T25 flask) (U.S.A. Scientific, Ocala, FL, USA) at a 45° angle for 7 days (37°C, 5% CO₂) with 10 U/ml of mIL-2 in cRPMI. On day 4, fresh cRPMI was added (1 μ g/ml of peptides and protein with 5 U/ml of IL-2 final concentration). On day 7, effector cells (splenocytes) were assayed for cytolytic activity in a non-radioactive CTL assay using two BC10ME cell lines stably expressing HIV-1 Gag (relevant protein) or Pol (irrelevant protein). Mouse splenocytes (1×10^5 - 8×10^6 effector cells) and BC10ME_{Gag or Pol} (1×10^5 target cells) were incubated in a 96 well plate (Corning, Corning, NY, USA) for 5 h at 37°C in 5% CO₂. Cytotoxicity was measured quantitatively by the release of lactate dehydrogenase (LDH) from lysed cells using a CytoTox 96 Non Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA).

After 5 h, the samples were centrifuged (3,000 rpm for 10 min), and 100 µl of culture supernatant was added to each well of a new 96 well plate. Cell Titer 96[®] AQueous One Solution Reagent was thawed at 37°C for 10 min and pipetted (20 µl) into each well of the 96-well plate containing 100 µl of the test samples and gently mixed. The plate was incubated (37°C, 5% CO₂) for 2 h. The colorimetric change was measured as the O.D. (490 nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The arithmetic mean plus the S.D. was recorded after the appropriate background values were subtracted from the test samples. The percentage of cytotoxicity was determined by the following formula: % Cytotoxicity = [(Experimental - Effector Spontaneous - Target Spontaneous) ÷ Target Maximum - Target Spontaneous] X 100.

II.H.5. Western Blot Analysis for Anti-Gag Antibodies

Sera from rabbits vaccinated with VLP DNA was collected at week 20 (see Section II.G.3), and antibody responses were determined by western blot analysis. Purified HIV-1_{SF2} p55 Gag protein (20 ng) (NIH ARRRP, Germantown, MD, USA) was loaded per well and electrophoresed through a 5-10% gradient SDS-polyacrylamide gel (see Section II.C.2). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, and serum from immunized rabbits (1:1500) was used as the primary antibody. HIV-1 specific antibodies were detected using goat anti-rabbit IgG conjugated to HRP (1:3,000) (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were visualized by enhanced chemiluminescence followed by autoradiography (see 2.3.2).

II.H.6. Proliferation Assay

Peripheral blood mononuclear cells (PBMCs) were isolated from immunized rabbits ¹³¹ (see Section II.G.3). PBMCs were plated at a concentration of 2×10^5 cells/well (96-well plate) in 200 μ l of cRPMI (Corning, Corning, NY, USA). PBMCs were stimulated with recombinant HIV-1_{LAI or BAL} Env_{gp120} or Measles F protein (NIH ARRRP, Germantown, MD, USA) (10 μ g/ml). Plates were incubated at 37°C in a 5% CO₂ atmosphere for 72 h and then pulsed with 1 μ Ci per well of [³H]-thymidine (DuPont NEN, Boston, MA, USA) in 50 μ l of cRPMI. The cells were then incubated for an additional 24 h (37°C in 5% CO₂), and the incorporation of [³H]-thymidine was determined by harvesting the cells, immobilizing the DNA to glass fiber membranes, and counting in a liquid scintillation counter. Each sample was harvested onto individual circular regions of a glass filter paper (Wallac, Turku, Finland) using a Tomtec full-plate harvester (Tomtec, Orange, CT, USA). Scintillation cocktail (Beckman, Columbia, MD, USA) (3.6 ml) was added to the filter in a sealed bag and counted with a Wallac 1450 Microbeta Plus liquid scintillation counter (EG&G Wallac, Milton Keynes, UK). Mean liquid scintillation results for triplicate wells were recorded as counts per minute (cpm). The arithmetic mean of three samples per group was determined \pm the S.D.

II.I. Statistical Analysis

Sample size calculation for paired t test was determined using the following parameters: power = 90, alpha = 0.05 and confidence interval = 90 using the delta-sigma

approach using the following equation (assuming 3 fold differences in antibody titer and cellular responses and normal population standard deviation=1): $n=[2(z_{1-\beta}+z_{1-\alpha/2})^2]/(\delta/\sigma)^2$.

All statistical analysis was done using the Student's *t* test and STATA software (STATA Corp, College Station, TX, USA).

III. Chapter 3: Specific Aims

III.A. Rationale of Research

Overall Design and Rationale

There are several advantages of a multi-gene virus-like particle DNA vaccine approach for HIV-1 compared to individual gene vaccines. This strategy offers a cost-effective, environmentally stable, easily manipulated alternative to other vaccine approaches. A multi-gene DNA vaccine has the capability of provoking both arms of the immune system, inducing neutralizing antibodies against conformational epitopes, and expressing multiple viral proteins.

Multi-epitope vaccine in a single component vaccine

The VLP vaccine constructs express various HIV-1 proteins. A diverse group of antigens offers multiple epitopes that can be presented to the immune system by an array of MHC class I and II molecules. DNA vaccines elicit both cell-mediated and humoral immune responses. Subsequently, HIV-1 proteins entering the endogenous or exogenous antigen pathways will be presented by MHC I or II, respectively. Multi-epitope vaccines are more likely than their single component counterparts to generate a broad-based immune response capable of clearing HIV-1 escape mutants. Lastly, a multi-gene vaccine is much more cost effective compared to the equivalent individual genes expressed on different plasmids for development and mass production for toxicity, safety and immunogenicity in future clinical human trials.

HIV-1 clade B/R5-tropic vaccine

The majority of individuals in the United States and northern Europe are infected

with HIV-1 clade B strains. The VLP vaccines express proteins derived from clade B virus strains. Within a clade, strains exhibit some antigenic differences but are relatively similar. Therefore if a vaccine proves successful, vaccines can be designed to represent other clades such as clade A/C (Africa) and clade E (Thailand).

The majority of individuals newly infected contract an R5-tropic strain of HIV-1. As these individuals progress in disease, more X4-tropic strains are isolated from these patients rather than the original R5-utilizing isolates. So, Envs from R5-tropic strains (ADA, R2, JR-FL) were used to represent the primary isolates that are currently circulating in human populations today. In addition, Env from an R5X4 strain (89.6), capable of using either CCR5 or CXCR4 for entry, were also included in this study.

Vaccines capable of entering antigen presenting cells

The production of virus-like particles allows the opportunity of Env-mediated entry of the particles into professional antigen presenting cells (APCs) such as macrophages and dendritic cells. Both cell types express CD4 and CCR5, which R5-tropic HIV strains can use for entry. The VLP vaccines contain either R5 or R5X4 Envs that can bind to APCs. The VLP can undergo uncoating following Env-mediated entry. Therefore, the viral proteins can be processed and presented to the immune system, thus enhancing the humoral response to Env.

Virus-like particle immunogen with natural conformation of Env

Antigens presented in their native conformation (e.g. particle) elicit more effective humoral immune responses compared to individual proteins (soluble Gag or

Env). In addition, many neutralizing antibodies are generated against conformational epitopes only present in the native form of the antigen. The multi-gene vaccine produces virus-like particles containing the Env in a trimer/multimeric conformation similar to that encountered during a natural infection of HIV-1

DNA immunizations by gene gun lead to direct transfection of the professional APCs. Conversely, intramuscular inoculation of vaccine plasmids results in transfection of muscle cells or keratinocytes, which serve as factories of antigens but do not act as antigen presenting cells. An immunogen capable of entering APCs will increase the efficiency of the vaccine to stimulate both humoral and cell-mediated immunity. Unlike soluble antigen, particulate immunogens can be engulfed by professional APCs and enter the cross-presentation pathway thereby enhancing cellular responses in addition to humoral responses.

Mucosal vaccination of particulate immunogens

HIV-1, similar to many other pathogens, is primarily transmitted at the mucosal surface. Yet, the majority of experimental vaccines against HIV-1 have been inoculated parenterally. Although an efficient systemic response develops, immunity at the mucosa after parenteral inoculation is negligible. In contrast, mucosal immunization can induce immune responses at both local and distal mucosal sites as well as systemic immunity. An ideal HIV/AIDS vaccine will most likely need to elicit robust immunity against multiple viral antigens in both systemic and mucosal immune compartments. Therefore, augmentation of the immune response at the mucosa by mucosally-administered vaccines may induce high titer anti-HIV immunity and is more likely to prevent viral infection and

the establishment of viral reservoirs compared to vaccines inoculated parenterally.

Compared to particulate antigens, intranasal vaccination of soluble proteins (in the absence of an adjuvant) induces low or undetectable immune responses in rodents and primates. VLPs can be phagocytosed by M cells in the nasal lumen and then directly deposited to the NALT via M cell transcytosis, which preferentially drains into cervical lymph nodes. This process induces strong local (NALT) and distant immune responses in both peripheral and mucosal immune compartments. Soluble antigens bypass the NALT and are directly fed into superficial lymph nodes by APCs in the nasal lumen resulting in a lower local immune response. Therefore, particulate antigens are better mucosal immunogens compared to soluble proteins.

Use of CpG ODNs

Synthetic oligodeoxynucleotides containing unmethylated CpG motifs are potent immune adjuvants, which accelerate and boost antigen-specific immune responses following systemic or mucosal vaccination. CpG ODNs directly stimulate B cells and dendritic cells, thereby promoting the production of Th1 and pro-inflammatory cytokines and the maturation/activation of professional antigen-presenting cells. Animal challenge models establish that protective immunity can be accelerated and magnified by co-administering CpG DNA with vaccines similar to other adjuvants such as CT or RANTES.

III.B. Specific Aim I

Goal: To construct and characterize human immunodeficiency virus-like particle vaccines and test for efficient protein expression and particle formation.

Hypothesis: A human immunodeficiency virus multi-gene DNA vaccine will produce virus-like particles.

Summary: In SPECIFIC AIM I, a single vaccine plasmid was constructed to express Gag, Pol, Env, Tat, Rev, and Vpu, and this plasmid was examined for protein expression and particle formation. The VLP vaccine constructs were derived from the HIV-1_{BH10} provirus. Safety mutations and deletions were introduced into the VLP DNA to generate a vaccine insert that was non-infectious. The 5' and 3' long terminal repeats, integrase, *vif*, *vpr* and *nef* were removed to further enhance the safety of the vaccine insert. Moreover, mutations were introduced into nucleocapsid, reverse transcriptase, and protease to severely restrict viral RNA packaging and to abolish RT, RNase H, and protease activity. The multi-gene vaccine insert was under the transcriptional control of the CMV-IE, while the expression of the genes is virally regulated. Envelopes from different Clade B strains of HIV-1 (ADA, R2, 89.6) were cloned into the VLP_{BH10} Gag-Pol backbone. Western blot and functional assays demonstrated efficient protein expression. The expressed VLPs were efficiently released from human cells and were similar in density and protein composition to wild-type virions as shown by density gradient ultracentrifugation and Western blotting. These replication-incompetent VLPs bound human CD4, and therefore they have the potential to bind and infect susceptible

cells *in vivo*. In addition, second and third generation VLP vaccines have been generated and analyzed for protein expression and particle formation. These modified VLP vaccines include the following: VLP vaccines with codon-optimized *env* sequences (ADA, R2 and JR-FL); VLP vaccines containing two copies of the Mason-Pfizer monkey virus constitutive transport element (MPMV CTE); SHIV VLP vaccines expressing SIV Gag-PR-RT and HIV Vpu, Env, Rev and Tat; and HIV and SHIV VLP vaccines without RT sequences.

Conclusion: Virally-regulated, multi-gene DNA vaccines express gag, pol, env, tat, rev and vpu gene products and produce virus-like particles.

III.C. Specific Aim II

Goal: To determine the relative immunogenicity of human immunodeficiency virus-like particles.

Hypothesis: Purified virus-like particles administered intranasally will elicit humoral and cellular immune responses systemically and mucosally.

Summary: In Specific Aim II, the goal of this study was to assess the immunogenicity of purified VLPs inoculated intranasally alone or co-inoculated with cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs). A robust immune response at the mucosal surface will be essential in preventing the spread and establishment of the virus

within the host. Mice were inoculated intranasally to induce HIV-specific immune response systemically and at distal mucosal sites. Purified virus-like particles were used as the inoculum because of inherent problems associated with expressing virally-regulated VLP DNA in rodents. Initially, a minimal response to Env was expected due to the low number of Env molecules incorporated in the VLPs. Consequently, VLPs were co-immunized with CpG ODNs to enhance the immune response to Env. Human immunodeficiency virus-like particles were expressed from the DNA plasmids constructed and characterized in Specific Aim I. Each vaccine was expressed using sequences encoding *gag*, *pol*, (HIV-1_{BH10}), *tat*, *rev*, *vpu*, and *env* (HIV-1_{ADA} or R2). Each plasmid efficiently induced the secretion of particles from primate cells (COS or 293T); however, no particles were secreted from murine cells (NIH 3T3). Therefore, particles were purified from the supernatants of African green monkey cells (COS) and used as immunogens. In this study, BALB/c mice were inoculated intranasally with purified human immunodeficiency virus-like particles (day 1 and weeks 3 and 6). VLPs were purified via transient transfection of COS cells followed by ultracentrifugation through 20% glycerol. Each VLP elicited specific immunity to HIV-1 antigens in both the systemic and mucosal immune compartments. Anti-Env antibodies were detected in the sera, as well as in the washes from harvested lungs, intestines, and vagina from immunized mice. In addition, Env and Gag specific IFN- γ -secreting splenocytes were elicited in the mice vaccinated with VLPs. Co-inoculation of CpG ODNs with VLPs significantly enhanced both sets of the immune response.

Conclusion: Systemic and mucosal immune responses to HIV-1 are enhanced by intranasal immunization of purified virus-like particles expressed from a virally-regulated multi-gene DNA vaccine.

III.D. Specific Aim III

Goal: To compare the elicitation of immune responses by the same Gag immunogen presented to the immune system in different forms.

Hypothesis: The same immunogen delivered to the immune system in different forms will induce a diverse set of immune responses. Purified Gag_{p55} VLPs will be more immunogenic compared to soluble Gag_{p55} protein. DNA expressing VLPs will elicit humoral and cellular immune responses, while DNA expressing intracellularly-contained VLPs will induce mainly cellular immunity.

Summary: In SPECIFIC AIM III, the goal of the study was to compare the elicitation of immune responses by the same Gag immunogen presented to the immune system in different forms. The *gag* gene of the human immunodeficiency virus type 1 (HIV-1) encodes for viral proteins that self-assemble into viral particles. The primary Gag gene products (capsid, matrix, and nucleocapsid) elicit humoral and cellular immune responses during natural infection, and these proteins are crucial immunogens in many HIV/AIDS vaccines. However, the structural form (particulate or free, soluble polypeptides) of these proteins may influence the immunity elicited during vaccination. In this study, mice

were inoculated with vaccines expressing HIV-1 Gag gene products *in vivo* as 1) DNA plasmids expressing Gag particles (pGag) or 2) these same proteins retained intracellularly (pGag_{ΔMyr}). In a separate experiment the immunity elicited by: 3) Gag_{p55} particles (purified *in vitro*) or 4) soluble Gag_{p55} proteins administered by intramuscular injection was evaluated. Enhanced cellular responses, but almost no anti-Gag antibodies, were elicited with intracellularly-retained Gag proteins. In contrast, particle secreting cells elicited both anti-Gag antibodies and cellular responses. Mice vaccinated with purified Gag_{p55} pseudovirions elicited robust humoral and cellular immune responses, which were significantly higher than the immunity elicited by soluble, non-particulate Gag_{p55} protein. Overall, particles of Gag effectively elicited the broadest and highest titers of anti-Gag immunity. The structural form of Gag influences the elicited immune responses and should be considered in the design of HIV/AIDS vaccines.

Conclusion: Diverse immune responses are elicited by the same Gag immunogen presented to the immune system in different forms. Purified Gag_{p55} VLPs are more immunogenic compared to soluble Gag_{p55} protein (humoral and cellular). DNA expressing VLPs elicit humoral and cellular immune responses, while DNA expressing intracellularly-contained VLPs induces mainly cellular immunity.

IV. Chapter 4: Specific Aim I

Characterization of DNA Vaccines Expressing a Non-Infectious Human Immunodeficiency Virus-Like Particle

This chapter was modified with permission from:

Young, KR, JM Smith, and TM Ross.

**Characterization of a DNA vaccine expressing human
immunodeficiency virus-like particles.**

***Virology*, 2004, Oct 1; 327(2): 262-72.**

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IV.A. Introduction

Approximately forty-two million people are infected with the human immunodeficiency virus type 1 (HIV-1) ²⁸⁶. The virus continues to spread to new populations adding to the total number of infected individuals. The use of HAART has enhanced both the longevity and quality of life for infected individuals by controlling viral replication ²⁷⁰. Despite the effectiveness of HAART, several drawbacks are accompanied with this treatment that limits its worldwide use. First, HAART does not protect patients against initial infection, nor does HAART clear viral infection. Second, treatment is expensive particularly for individuals living in developing nations. Third, there can be severe side effects, and patients on HAART have difficulties adhering to the drug regimens. Therefore, several preventive measures to combat the spread of HIV infection have focused on the development of safe, inexpensive, and efficacious vaccines.

Many of the successful viral vaccines administered to patients, such as vaccines developed for the measles or mumps viruses, consist of replication-competent virus that has been attenuated in order to prevent disease ^{157, 304}. The experimental use of live-attenuated simian immunodeficiency virus (SIV) elicited protective immunity in non-human primates challenged with SIV or SHIV (a hybrid virus containing the env, tat vpu, and rev gene sequences from HIV in a SIV gag-pol backbone) infection ^{354, 483, 698}. In addition, these monkeys had specific cell-mediated and humoral immunity that appeared to correlate with protection. However, reversion of these vaccine strains from attenuated to pathogenic forms resulted in uncontrolled replication of the virus, pathogenesis, and subsequently death of the vaccinated animals. These results observed in non-human primates may have a direct bearing on the use of live-attenuated HIV for vaccination

against infection in humans. Consequently, this approach is not considered a viable vaccine strategy for preventing HIV/AIDS.

The development of DNA vaccine (genetic vaccine) technology has opened new avenues for AIDS vaccine research. These genetic vaccines consist of eukaryotic expression plasmids that are inoculated into target cells of a vaccinated host and subsequently translated into protein¹⁵². DNA plasmids inoculated *in vivo*, expressing a gene insert, elicit protective immunity against a variety of pathogens^{152, 542, 543}. Similar to live-attenuated virus, DNA vaccines expressing lentiviral antigens elicit both high titer antibody and cell-mediated immune responses to HIV/SIV in both rodents and non-human primates^{328, 490}. Therefore, administration of DNA plasmid is an alternative strategy to the use of live-attenuated virus.

The induction of a broad range of immune responses appears necessary for any vaccine strategy against HIV/AIDS⁶¹⁰. A predominately antibody-mediated immune response does not confer protection against HIV infection⁴²⁰. Non-human primates vaccinated with plasmids expressing HIV or SIV antigens had high-titer, anti-viral antibodies but were unable to control viral challenge^{468, 675}. In pre-clinical human trials, volunteers vaccinated with recombinant Env_{gp120} or Env_{gp160} elicited transient, non-neutralizing anti-Env antibodies that did not result in long-term immune memory³⁸⁹. However, an exclusively cell-mediated immune response against HIV/SIV antigens also does not appear to correlate with long-term protective immunity¹⁴. Vaccine strategies that elicit both humoral and cell-mediated immunity appear to be crucial in limiting viral replication and protection from live virus infection. Monkeys primed with DNA expressing Gag-Pol and Env, followed by a vector boost of modified vaccinia Ankara

(MVA) expressing the same antigens, were able to control viral replication more effectively than those animals vaccinated with only Gag-Pol¹⁴. These results highlight the importance of immune responses to Env, as well as to Gag-Pol, in controlling immunodeficiency virus challenges. In the present study, a DNA vaccine was constructed to express a virally-regulated human immunodeficiency virus-like particle in order to elicit broad-spectrum immune responses to multiple HIV-1 antigens. These VLP were engineered with specific safety mutations and deletions in the genome to prevent integration, severely restrict RNA genome packaging, as well as inhibit reverse transcriptase and protease activity.

IV.B. Material and Method

DNA plasmids from external sources

The lineage of all vaccine plasmids is described in Table 5. The vector plasmid, pTR600^{62, 235, 446, 634, 720}, (Accession number: AF425297) (Harriet Robinson, Emory University, Atlanta, GA, USA) uses the cytomegalovirus immediate-early (CMV-IE) promoter to efficiently initiate transcription (Figure 6). Sequences from the bovine growth hormone terminator (BGH Poly A Term) were inserted to provide a polyadenylation signal. The vector also contains the Col E1 origin of replication for prokaryotic replication, as well as the kanamycin resistance (Kan^r) gene for selection in antibiotic media.

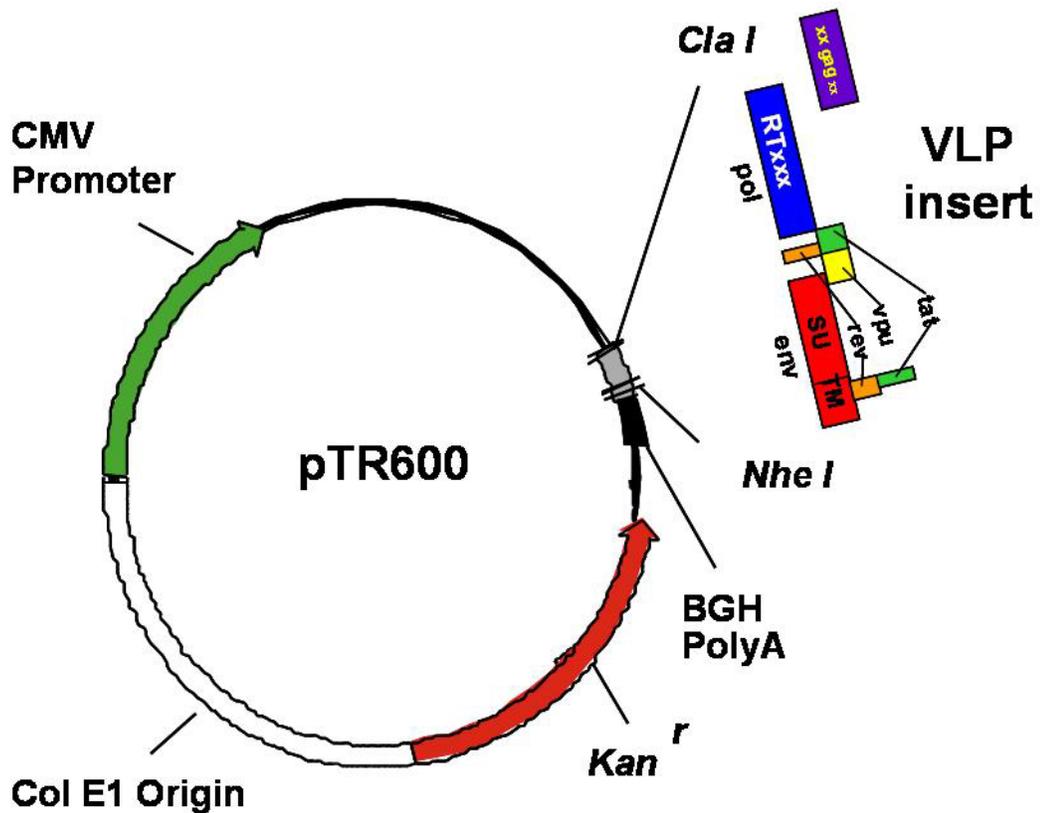


Figure 6. Schematic of VLP DNA vaccines.

The plasmid, pTR600, was constructed to contain the 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. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (*Kan^r*) for selection in antibiotic media. VLP sequences and other genes of interest were cloned into the multiple cloning site (MCS).

Table 5. Summary of molecular cloning of vaccine plasmids.

Plasmid	Vector Parent	Insert Parent	Restriction Endonucleases (insert/vector)	Cloning Verification by Restriction Endonuclease Digestion
pVLP _{ADA}	pVLP _{BH10}	pHIV-1 _{ADA}	<i>EcoR I-Nhe I</i> (3.1 kb/6.5 kb)	<i>Avr II</i> 800 bp/4.2 kb/4.5 kb correct 4.1kb/5.4kb wrong
pVLP _{89.6}	pVLP _{ADA}	pHIV-1 _{89.6}	<i>EcoR I-Bam HI</i> (2.7 kb/6.8 kb)	<i>EcoR I/Nde I</i> 2.2 kb/7.3 kb correct 9.5 kb wrong
pVLP _{R2}	pVLP _{ADA}	pR2 _{gp160}	<i>Xho I-Bam HI</i> (2.2 kb/7.3 kb)	<i>EcoR V</i> 2.4 kb/7.1 kb correct 9.5 kb wrong
pVLP _{JR-FL}	pVLP _{ADA}	pHIV-1 _{JR-FL}	<i>Xho I-Bam HI</i> (2.2 kb/7.3 kb)	<i>EcoR V</i> 2.4 kb/7.1 kb correct 9.5 kb wrong
pVLP _{coADA}	pVLP _{ADA}	pcoADA _{gp160}	<i>Eco RI-Bam HI</i> (2.7 kb/ 6.8 kb)	<i>Eag I/Bam HI</i> 1.3 kb/8.2 kb correct 9.5 kb wrong
pVLP _{coR2}	pVLP _{ADA}	pcoR2 _{gp120}	<i>Xho I-Mlu I</i> (1.5 kb/8.0 kb)	<i>Apa I</i> 3.7 kb/5.8 kb correct 9.5 kb wrong
pVLP _{ADA/2xCTE}	pVLP _{ADA}	pCDNA-gp3v-CTE-CTE	<i>Nhe I-Sac II</i> (550 bp/9.5 kb)	<i>Nhe I-Sac II</i> 550 bp/9.5 kb correct 27 bp/9.5 kb wrong
pVLP _{ADA/ART}	pVLP _{ADA}	n/a	<i>Eco RI</i> (3.6 kb/5.9 kb)	<i>EcoR I</i> 7.9 kb correct 1.7 kb/7.9 kb wrong
pVLP _{SHIV/ADA}	pVLP _{ADA}	pSIV _{mac239-3'}	<i>Mlu I-Eco RI</i> (3.5 kb/6.0 kb)	<i>Bam HI</i> 3.8 kb/5.7 kb correct 9.5 kb wrong
pVLP _{SHIV/ADA/ART}	pVLP _{SHIV/ADA}	n/a	<i>Eco RI</i> (1.7 kb/7.8 kb)	<i>EcoR I</i> 7.9 kb correct 1.7 kb/7.9 kb wrong
pCD4	pTR600	sT4DHFR	<i>Hind III/Nhe I</i> (1.2 kb/3.9 kb)	<i>Hind III/Nhe I</i> 1.2 kb/3.9 kb correct 3.9 kb wrong
pcoADA _{gp120}	pTR600	pcoADA _{gp160}	<i>Hind III/Bam HI</i> (1.4 kb/3.9 kb)	<i>Hind III/Bam HI</i> 1.4 kb/3.9 kb correct 3.9 kb wrong
pcoR2 _{gp120}	pTR600	pGAcoR2 _{gp120}	<i>Hind III/Bam HI</i> (1.4 kb/3.9 kb)	<i>Hind III/Bam HI</i> 1.4 kb/3.9 kb correct 3.9 kb wrong
pGag	pTR600	phGag	<i>Hind III-Bam HI</i> (1.5 kb/3.9 kb)	<i>Hind III-BamHI</i> 1.5 kb/3.9 kb correct 3.9 kb wrong
pGag-Pol	pTR600	phGag-Pol	<i>Cla I- Eco RI</i> (4.3 kb/3.9 kb)	<i>Cla I- Eco RI</i> 4.3 kb/3.9 kb correct 3.9 kb wrong

The vector plasmid, pBC12¹²⁰, (Brian Cullen, Duke University, Durham, NC, USA) contains the CMV-IE promoter for efficient initiation of transcription. Sequences from the bovine growth hormone polyadenylation signal were inserted to promote termination. The vector also contains the Col E1 origin of replication for prokaryotic replication as well as the ampicillin resistance (Amp^r) gene for selection in antibiotic media.

The following plasmids were generously donated to our laboratory: pHIV-1_{ADA} (HIV-1_{BH10} proviral backbone with HIV-1_{ADA} VERT region in pCB12 vector)^{104, 120, 406}, pHIV-1_{BH10} (pBluescript II-KS + vector, Stratagene, La Jolla, CA, USA)^{275, 530} and pHIV-1_{89.6} (pUC19 vector)^{48, 112, 153, 326} (National Institutes of AIDS Health Research and Reference Reagent Program (NIH ARRRP), Germantown, MD, USA). Each plasmid contains the full length HIV-1 provirus (BH10, ADA/BH10 hybrid and 89.6 isolates, respectively) with intact 5' and 3' LTRs.

The reporter plasmid, pBlueScript3-HIV-LTR-SEAP (pLTR-SEAP), expresses secreted alkaline phosphatase from the promoter found in the HIV-1 5' long terminal repeat (LTR) (Brian Cullen, Duke University, Durham, NC, USA)⁴⁰.

Construction of VLP vaccines

The construction of the VLP vaccines is outlined in Table 2 and Figure 7. The VLP parental plasmid, pHIV-1_{BH10}, encoded the sequences for the proviral HIV-1 from the isolate BH10 (X4) (Accession number: M1564) (pBC12) and was used as a template to construct the plasmid, pVLP_{BH10/wt}. pVLP_{BH10/wt} encodes for the following gene sequences: *gag* and *pol* (HIV-1_{BH10} nt 1-3626, VLP_{ADA} nt 7-3620) *vpu*, *env*, *rev* and *tat*

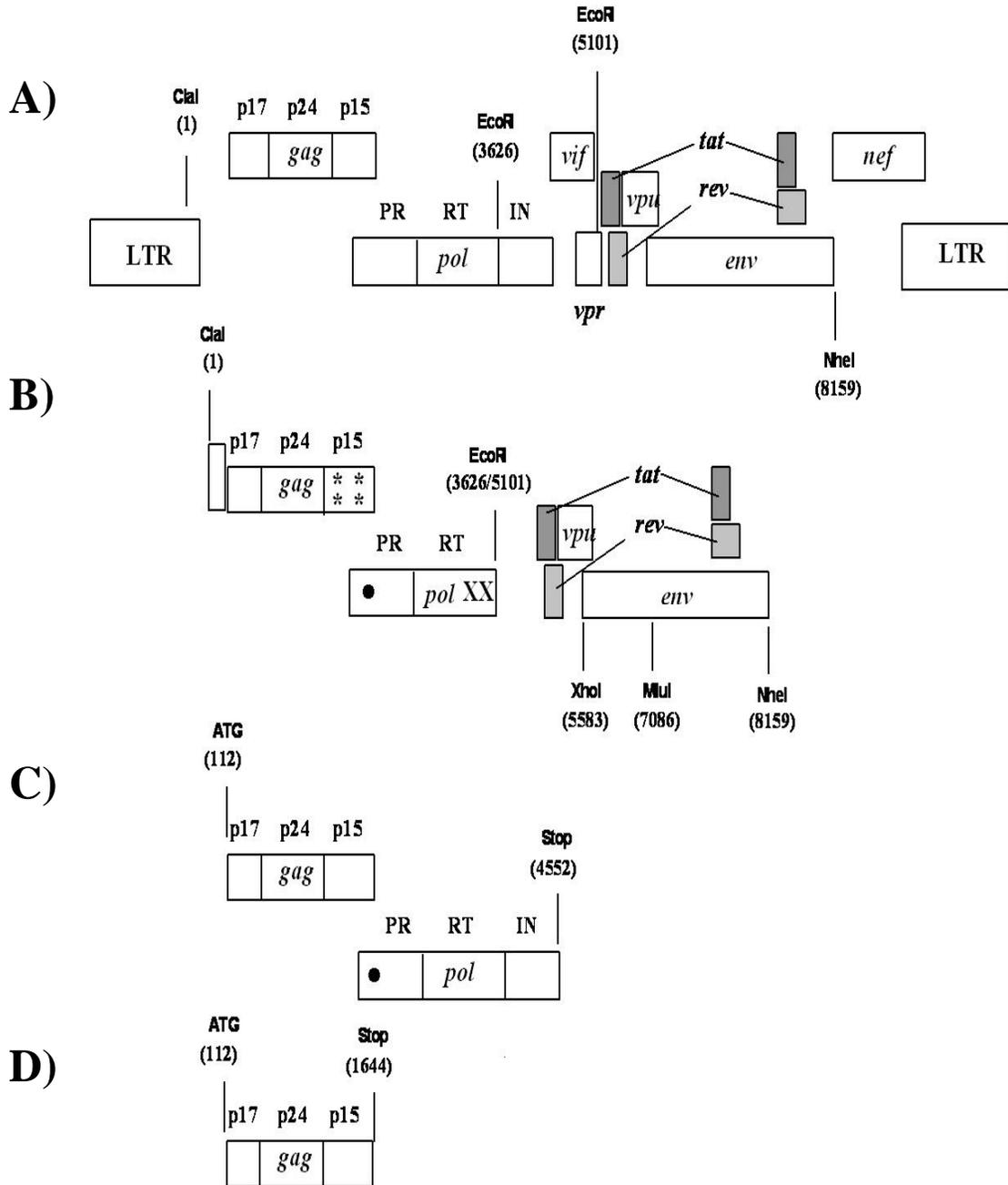


Figure 7. Schematic representation of DNA vaccine constructs.

(A) Wild-type, proviral DNA, (B) VLP DNA with regions *env* encoding for the 5' PCR product (HIV-1_{BH10}: p17, p24, p15, PR, RT) and the 3' PCR product (HIV-1_{ADA}, 89.6, or R2: *vpu*, *env*, *rev*, *tat*), (C) Gag-Pol DNA, (D) Gag DNA. Restriction sites are based on the nucleotide sequence for HIV_{BH-10}. * represent the mutations in NC. • represents the mutations in PR. X represents the mutations in RT.

(HIV-1_{BH10} nt 5101-8160, VLP_{ADA} nt 3620-6672). The pVLP_{BH10} plasmid was constructed by first making two subclones encoding for the 5' end of the VLP gene insert and a second subclone encoding for the 3' end of the VLP gene insert. Oligonucleotides corresponding to the *gag-pol* sequences were used to amplify a fragment of DNA (5' PCR product) composed of the 5' untranslated leader sequence (105 nucleotides) and *gag-pol* sequences (start codon of Gag to the stop codon of RT) by PCR (see Section II.A.5). For the 5' PCR product, a *Cla I* restriction enzyme site was introduced (HIV-1_{BH10} nt 7, VLP_{ADA} nt 7) at the 5' end of the vaccine insert, while *EcoR I* (HIV-1_{BH10} nt 3626, VLP_{ADA} nt 3620) and *Nhe I* sites (HIV-1_{BH10} nt 8160, VLP_{ADA} nt 6672) were added at the 3' end of the 5' PCR product by PCR. The following primers were used to amplify the 5' PCR product (3625 bp): (sense primer 5'-GAGCTCTATCGATGCAGGACTCGGCTTGC-3' and antisense primer 5'-GGCAGGTTTTAATCGCTAGCCTATGCTCTCC-3'). The 3' PCR fragment (3059 bp) encoded the *vpu*, *tat*, *rev*, and *env* gene sequences. An *EcoRI* site was introduced into the 5' terminus of the 3' PCR product (HIV-1_{BH10} nt 5101, VLP_{ADA} nt 3620) and an *Nhe I* site was introduced at the 3' terminus (HIV-1_{BH10} nt 8160, VLP_{ADA} nt 6672), and the 3' PCR product was amplified by the following primers: (sense primer 5'-CCCACCTTAAGACGTTGTTGACGACAAATACG-3' and antisense primer 5'-CCACACTACTTTCGGACCGCTAGCCACCC-3'). The PCR products were cloned into pCR2.1-TOPO (Invitrogen Life Sciences, Carlsbad, CA, USA) (see Section II.A.5). Both the 5' and 3' PCR products were cloned into pTR600 using unique restriction enzyme sites (5' PCR product: *Cla I* and *EcoRI* and the 3' PCR product using *EcoRI* and *Nhe I*) described in Sections II.A.1 and II.A.2. The gene inserts were verified by digestion with

restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{BH10} is derived from the plasmid, pVLP_{BH10/wt} (see Table 2). Safety mutations were introduced in pVLP_{BH10/wt} in the zinc fingers in nucleocapsid (NC) to inhibit RNA packaging (C15S, C36S)⁴⁴⁹. In addition, mutations were introduced into the *pol* gene to abrogate RT activity (D185N)⁹⁵, inhibit strand transfer (W266T)³⁶⁸, and to inactivate RNase H activity (E478Q)⁷⁰⁷. All mutations were introduced by SDM PCR (see Section II.A.6 and Table 6) and verified by DNA sequencing (see Section II.A.7).

Three additional VLP DNA plasmids were constructed that encoded for various *env* sequences. The *env*, *tat*, *vpu* and *rev* sequences were cloned by PCR amplification from the DNA from the ADA (Bryan Cullen, Duke University, Durham, NC, USA), 89.6 (NIH ARRRP, Germantown, MD, USA), R2 (Gerald Quinnan, USA), or JR-FL (Shan Lu, University of Massachusetts, Worcester, MA, USA) isolates of HIV-1 and cloned into the subclone, pCR2.1-TOPO (Invitrogen Life Sciences, Carlsbad, CA, USA) (see Section II.A.5). Each PCR fragment (2.7 kb, *env*, *tat*, *vpu* and *rev* sequences) was inserted into the VLP_{BH10} Gag-Pol backbone using unique restriction enzyme sites (see Sections II.A.1, II.A.2 and Table 5). The gene inserts were verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{89.6} was used as a model for the method of molecular cloning (Figure 8). VLP_{89.6} was constructed by restriction endonuclease digestion of parental vector (pVLP_{ADA}) and insert (pHIV-1_{89.6}) DNA. The parental plasmids, pVLP_{ADA} (500 ng) and pHIV-1_{89.6} (1 µg), were digested with restriction endonucleases, *EcoR I* and *Bam HI* (1 U each) for 2 h (see Section II.A.1). The digested products were resolved on a 1% agarose gel by electrophoresis. The parental insert fragment (2.7 kb) was extracted and purified

Table 6. Primers used to introduce mutations to inactivate protein activity by site-directed mutagenesis polymerase chain reaction.

Mutation	Nucleotide Sequence (5'-3')	Location[†]
C15S	ggttaagagcttcaatagcggcaaagaagggc gccctctttgccgctattgaagctcttaacc	1285
C36S	gggcagctggaaaagcggaaaggaagg ccttcctttccgctttccagctgccc	1357
D185N	ccagacatagttatctatcaatacatgaacgattgtatgtagg cctacatacaaatcggtcatgtattgatagataactatgtctgg	2460
W266T	ggggaaattgaataaccgcaagtcagatttacc gggtaaatctgacttgccgtattcaattcccc	2703
E478Q	ccctaactaacacaacaaatcagaaaactcagttacagc gcttgtaactgagtttctgatttgtgtgtagtaggg	3339
D25N	ggaagctctattaaatacaggagcagatg catctgctcctgtatttaatagagcttcc	1683
G2A	Gag myristylation	

[†]Location of the first nucleotide in the codon that has been changed in the HIV-1_{III_B} sequence

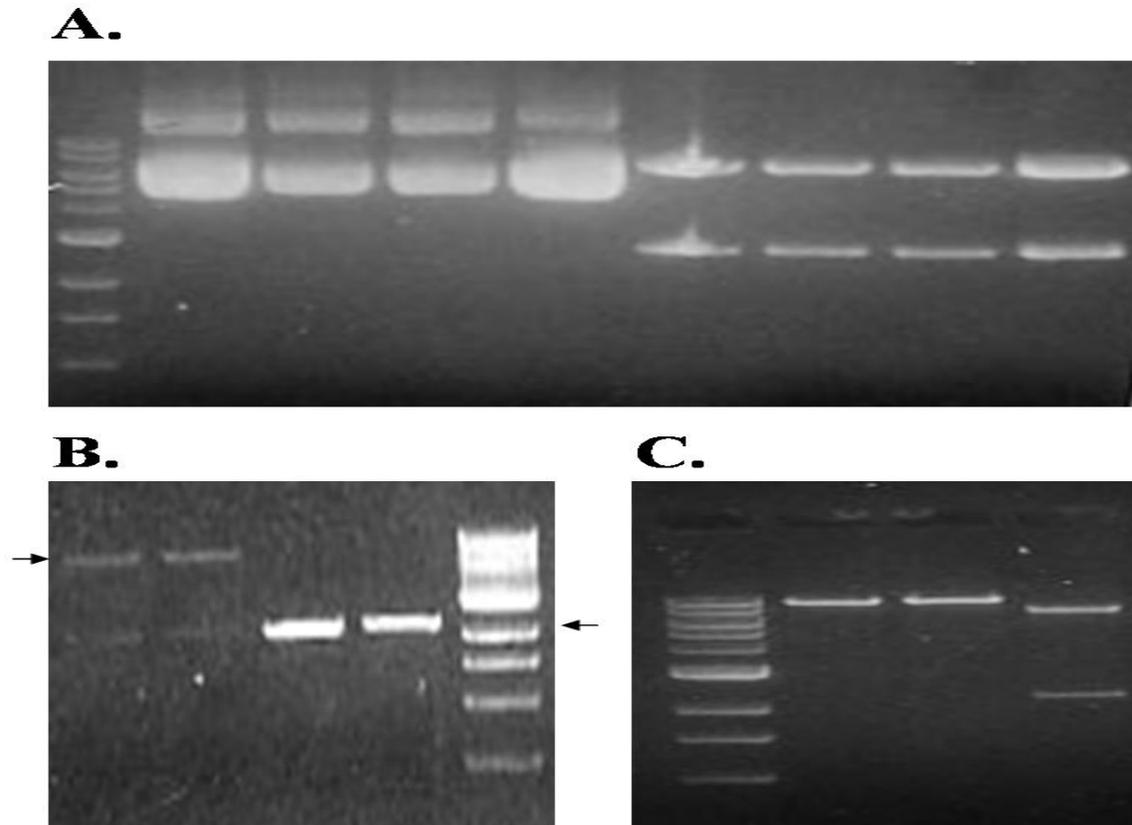


Figure 8. Molecular cloning of pVLP_{89.6}.

(A) Restriction endonuclease digestion of parental vector (pVLP_{ADA}) and insert (pHIV-1_{89.6}). The parental plasmids, pVLP_{ADA} (500 ng) and pHIV-1_{89.6} (1 µg), were digested with restriction endonucleases, *EcoR I* and *Bam HI* for 2 h. The digested products were resolved on a 1% agarose gel by electrophoresis. Lane 1, 1.0 kb ladder; lanes 2, 3, uncut pVLP_{ADA}; lanes 4, 5, uncut pHIV-1_{89.6}; lanes 6, 7, pVLP_{ADA} (*EcoR I/Bam HI*); lanes 8, 9, pHIV-1_{89.6} (*EcoR I/Bam HI*). (B) Agarose gel extraction and purification of parental insert fragment. The parental insert fragment (2.7 kb) was extracted and purified from the agarose gel to be used for ligation reaction. The parental vector fragments (2.7 kb/6.8 kb) (10% of total purified fragment) and the purified parental insert fragment (2.7 kb) (25% of digestion reaction from (A)) were resolved on a 1% agarose gel by electrophoresis. Lanes 1, 2, pVLP_{ADA} (2.7 kb/6.8 kb, vector), lanes 3, 4, pHIV-1_{89.6} (2.7 kb, insert); lane 4, 1.0 kb ladder. Arrows indicate the insert (right) and vector (left) fragments of interest. (C) Verification of molecular cloning of pVLP_{89.6}. The parental vector fragments and purified parental insert were ligated, transformed into XL-10 Gold cells and plated on antibiotic media. Plasmid DNA was purified from bacterial cells, digested (300ng) with *EcoR I/Nde I* (2 h) to verify molecular cloning and resolved on a 1% agarose gel by electrophoresis. Lane 1, 1.0 kb ladder, lane 2, pVLP_{ADA} (*EcoR I/Nde I*); lane 2, pVLP_{89.6} clone 1 (*EcoR I/Nde I*); lane 3, pVLP_{89.6} clone 2 (*EcoR I/Nde I*). The digested products were analyzed for fragment size (2.2 kb/7.3 kb, correct; 9.5 kb, wrong). pVLP_{89.6} clone 2 is correct and was amplified and purified from bacterial cells for further characterization.

from the agarose gel and used for the ligation reaction (see Section II.A.4). The parental vector fragments (2.7 kb/6.8 kb) (10% of total purified fragment) and the purified parental insert fragment (2.7 kb) (25% of digestion reaction) were resolved on a 1% agarose gel by electrophoresis. The parental vector fragments and purified parental insert were ligated, transformed into XL-10 Gold cells and plated on antibiotic media (see Section II.A.2 and II.A.3). Plasmid DNA was purified from bacterial cells, digested (300ng) with *EcoR I/Nde I* (2 h) to verify molecular cloning and resolved on a 1% agarose gel by electrophoresis. The digested products were analyzed for fragment size (2.2 kb/7.3 kb, correct; 9.5 kb, incorrect). The correct clone was amplified and purified from bacterial cells for further characterization.

Two other VLP-expressing DNA plasmids were constructed that contained codon optimized *env* sequences (GENEART, Regensburg, Germany). pVLP_{coADA} is derived from the plasmid, pVLP_{ADA}. The codon-optimized sequences representing the HIV-1_{ADA} *env* gene (pcoADA_{gp160}) was cloned into the plasmid, pVLP_{ADA}, using unique *EcoR I* (HIV-1_{BH10} nt 3626) and *Bam HI* (HIV-1_{BH10} nt 6349) restriction enzyme sites (see Section II.A.1 and II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{coR2} is derived from the plasmid, pVLP_{ADA}. The codon-optimized *env* sequence from HIV-1_{R2} (pGAcoR2_{gp120}) was cloned into the plasmid, pVLP_{ADA}, using unique *Xho I* (HIV-1_{BH10} nt 4111, VLP_{ADA} nt 4105) and *Mlu I* (HIV-1_{BH10} nt 5588, VLP_{ADA} nt 5582) restriction enzyme sites (see Sections II.A.1 and II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{ADA/2xCTE} was derived from the plasmid, pVLP_{ADA}. Two tandem copies of the Mason-Pfizer Monkey Virus (MPMV) constitutive transport element (CTE) (2xCTE) were cloned by PCR amplification from the plasmid, pCDNA-gp3v-CTE-CTE^{611, 614} and cloned into pCR2.1-TOPO (sense primer 5'-ctgcgaccgcggtgggagtgggcaccttcagggtc-3' and antisense primer 5'-gcggtgctagccaccataatcgcgccgctcg-3') (see Section II.A.5). The 2xCTE fragment was cloned into pVLP_{ADA} using the unique restriction sites, *Nhe I* and *Sac II* (see Section II.A.1 and II.A.2. The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{R2/ΔRT} is derived from the plasmid, pVLP_{R2}. Introduction of an *EcoR I* restriction enzyme site into pVLP (HIV-1_{BH10} nt 1937) was performed by SDM PCR (sense primer 5'-gagactgtaccagtgaattcaaagccaggaatggatggccc-3' and antisense primer 5'-gggccatccattctggtttgaattcaactggtacagtctc-3') (see Section II.A.6). pVLP_{R2} with an *EcoR I* site (500 ng) was digested with *EcoR I* (see Section II.A.1) and re-ligated to remove the sequences encoding RT (HIV-1_{BH-10} nt 1937-3626, 1.7 kb fragment) (see Section II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

Construction of SHIV VLP vaccines

pVLP_{SHIV/ADA} is derived from the plasmid, pVLP_{ADA}. Introduction of a *Mlu I* restriction enzyme site into pVLP_{ADA} (SIV_{mac239} nt 1313, HIV-1_{BH-10} nt 135) was performed by SDM PCR (sense primer 5'-gcgagagcgtcagtattacgcgtgggagaattagatcg -3' and antisense primer 5'-cgatctaattctcccacgcgtaatactgacgctctcgc-3') (see Section II.A.6 and Table 4). Sequences encoding for *gag*, PR, and RT from SIV_{mac239} were cloned by

PCR amplification from the plasmid, pSIV_{mac239-5'}, and cloned into pCR2.1-TOPO (Invitrogen Life Sciences, Carlsbad, CA, USA) (sense primer 5'-*ctacgtacgcgtgaagaaagcagatg-3'* and antisense primer 5'-*gcatggaattctaatacatgttcttctgtgc-3'*) (see Section II.A.5). The gene insert was cloned into pVLP_{ADA} using the unique restriction sites, *Mlu I* and *Eco RI*. This plasmid contains the sequences for *gag*, PR, RT (SIV_{mac239}) *env*, *vpu*, *tat* and *rev* (HIV-1_{ADA}). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pVLP_{SHIV/ADA/ART} is derived from the plasmid, pVLP_{SHIV/ADA}. An *Eco RI* restriction enzyme site was introduced into pVLP_{SHIV/ADA} (SIV_{mac239} nt 3131, VLP_{ADA} nt 1929) by SDM PCR (sense primer 5'- *gtagagcctgtgaattccgccttaaagccagg -3'* and antisense primer 5'- *cctggctttaaggcggaattcacaggctctac -3'*). The plasmid was digested with *Eco RI* and religated to remove the sequences encoding RT (HIV-1_{BH-10} nt 3133-4822, 1.7 kb fragment). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

Construction of HIV-1 Gag/Gag-Pol vaccines

pGag and pGag-Pol are derived from the codon optimized sequences encoding HIV-1_{NL4-3} *gag* (phGag) and *gag-pol* (phGag-Pol), respectively (generously donated by Gary Nabel, NIH, Bethesda, MD, USA)²⁸³. Sequences encoding for Gag_{p55} (pHIV_{BH10}, nt 112-1644) and Gag-Pol_{p160} (pHIV_{BH10}, nt 112-4552) were molecularly cloned from hGag and hGag-Pol into pTR600 using the unique restrictions sites, *Hind III/BamH I* and *Hind III/Eco RI*, respectively (see Section II.A.1 and II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

The plasmid, pGag_{ΔMYR}, is derived from the plasmid, pGag, and was constructed by mutating the myristylation site (second amino acid in MA_{p17}) by SDM PCR (sense primer 5'-cgtcgtcgacatggccgcccgcgccagcgtgc-3' and antisense primer 5'-gcacgtggcgcgggggccatgtcgacgacg-3') (see Section II.A.6). This mutation resulted in a change of the codon from a glycine to an alanine (gga to gca), which was verified by DNA sequencing (see Section II.A.7)

Construction of codon-optimized HIV-1 Env

pcoADA_{gp120} contains the codon-optimized sequences encoding the soluble Env_{gp120} from HIV-1_{ADA} (HIV-1_{ADA} Env: aa 1-520)⁷²¹. The codon-optimized Env_{gp120} gene was cloned by PCR amplification from the plasmid, pcoADA_{gp160}, (generous gift from Harriet Robinson, Emory University, Atlanta, GA, USA) and cloned into pCR2.1-TOPO (Invitrogen Life Sciences, Carlsbad, CA, USA) (sense primer 5'-gagcgtgctagcctgtgggtgaccgtgtactac-3' and antisense primer 5'-cgtgagggatcctgccttggtgggtgctactcc-3') (see Section II.A.5). The gene insert was cloned into pTR600 using the unique restriction sites, *Hind III* and *BamH I* (see Section II.A.1 and II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

pcoR2_{gp120} contains the codon-optimized sequences encoding the soluble Env_{gp120} from HIV-1_{R2} (HIV-1_{R2} Env: aa 1-520). The codon-optimized Env_{gp120} gene was molecularly cloned from the plasmid, pGAcoR2_{gp120}, (GENEART, Regensburg, Germany) into pTR600 using the unique restriction sites, *Hind III* and *BamH I* (see Section II.A.1 and II.A.2). The gene insert was verified by digestion with restriction

endonucleases (see Section II.A.1 and Table 5).

Construction of soluble human CD4

pCD4 expresses the soluble human CD4 (4 extracellular domains) (aa 1-370) using sequences from the full length CD4 gene (sT4DHFR) (Bryan Cullen, Duke University, Durham, NC, USA)^{105, 136, 406, 407, 432}. Soluble hCD4 was cloned by PCR amplification into pCR2.1-TOPO (Invitrogen Life Sciences, Carlsbad, CA, USA) (sense primer 5'-*gtcagcaagcttatgaaccggggagtcc*-3' and antisense primer 5'-*gctgacgctagegctacctcctccgctccatcgatgctacctccgctccgctacctccgcttccgaattcggtggaccattgtg*-3') (see Section II.A.5). The gene insert was cloned into pTR600 using unique restriction sites, *Hind III* and *Nhe I* (see Sections II.A.1 and II.A.2). The gene insert was verified by digestion with restriction endonucleases (see Section II.A.1 and Table 5).

Transfections and expression analysis

The monkey fibroblast cell line, COS, (5×10^5 cells/transfection) was transfected with 2 μ g of DNA using 12% lipofectamine according to the manufacturer's guidelines (Life Technologies, Grand Island, NY). Supernatants (2 ml) were collected and stored at -20°C . Cell lysates were collected in 500 μ l of 1% Triton X-100 and stored at -20°C . Quantitative antigen capture ELISAs were conducted according to the manufacturer's protocol (Perkin Elmer Life Sciences, Boston, MA).

For Western hybridization analysis, 3.3% of supernatant and 1.5% of the cell lysate was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA), boiled for 5 min, and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred

onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with a 1:5,000 dilution of polyclonal human HIV-infected patient antisera (HIV-Ig) in PBS containing 0.05% Tween 20 and 5% non-fat dry milk. After extensive washing, bound human antibodies were detected using a 1:7,000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

For determination of Tat activity, COS cells (5×10^5) were transiently transfected using 12% lipofectamine in DMEM with 1 μ g of each VLP DNA and 1 μ g of the reporter plasmid, pLTR-SEAP¹¹⁸. After 18 hours, cells were replenished with fresh media (1.5 ml) and incubated an additional 48 h. 100 μ l of collected supernatants were incubated with 100 μ l of SEAP buffer (2M diethanolamine, 1mM MgCl₂, 20mM L-homoarginine) for 10 min at 37 °C followed by the addition of 20 μ l p-nitrophenyl phosphate (120 mM) (PNP) (Sigma, St. Louis, MO, USA) for 30 min at 37°C. Samples were analyzed for color change at 405 nm. Data was recorded as the average of the three independent experiments +/- standard deviation.

Virus-like particle formation

Supernatant from COS cells, transiently transfected with plasmid expressing Gag, Gag-Pol, HIV/SHIV VLP, or infectious virions, were pelleted via ultracentrifugation (100,000 x g through 20% glycerol) for 2 h at 4°C. The pellets were subsequently resuspended in PBS and overlaid onto 20-60% sucrose gradients (11 steps, 4% increments) and ultracentrifuged for 17 h at 100,000 x g at 4°C. Eleven fractions (20-60%, 1ml) were collected top to bottom from the gradient, and the proteins were

precipitated with equal volumes of 20% TCA and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by HIV Ig or SIV Ig via Western hybridization.

VLP binding to human CD4

Supernatants from COS cells transiently transfected with plasmids expressing either pTR600, pVLP, pHIV, or pGag-Pol were incubated at RT for 4 h with supernatants from COS cells transiently transfected with a plasmid expressing soluble human CD4 (sCD4) (9:1 ratio). The mixture was centrifuged (100,000 x g) and pelleted through 20% sucrose. Each pellet was resuspended in PBS and analyzed by Western hybridization for CD4 bound to VLP. sCD4 was detected by mouse polyclonal anti-CD4 (1:3,000) followed by goat anti-mouse IgG conjugated to HRP (1:7,000) (Bio-Rad, Hercules, CA). The proteins were enhanced by chemiluminescence and visualized by autoradiography.

Rabbit immunizations

Five to 7 week old New Zealand white rabbits were vaccinated (i.m.) at weeks 0, 6, 12 and 18 with 200 µg of VLP DNA (pVLP_{89,6}) in 500 µl of sterile PBS. The rabbits were housed in compliance with the USDA regulations. Rabbits were monitored daily for weight loss, behavior, and adverse reaction.

Western blot analysis for anti-Gag antibodies

Sera from rabbits vaccinated with VLP DNA were collected at week 20, and antibody responses were determined by Western blot analysis. Purified HIV-1_{SF2} Gag_{p55}

protein (20 ng) (NIH ARRRP) was loaded per well and electrophoresed through a 5-10% gradient SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane, and serum from immunized rabbits (1:1500) was used as the primary antibody. HIV-1 specific antibodies were detected using goat anti-rabbit IgG conjugated to HRP (1:3,000). The proteins were visualized by enhanced chemiluminescence followed by autoradiography.

Anti-Env proliferation responses

PBMCs were isolated from immunized rabbits 25 weeks following the last immunization (week 43)¹³¹. PBMCs were plated at a concentration of 2×10^5 cells/well (96-well plate) in 200 μ l of cRPMI. PBMCs were stimulated with recombinant HIV-1_{LAI} or BAL Env_{gp120} or Measles F protein (NIH ARRRP) (10 μ g/ml). Plates were incubated at 37°C in a 5% CO₂ atmosphere for 72 h and then pulsed with 1 μ Ci per well of [³H]-thymidine in 50 μ l of cRPMI. The cells were then incubated for an additional 24 h (37°C in 5% CO₂), and the incorporation of [³H]-thymidine was determined by harvesting the cells, immobilizing the DNA to glass fiber membranes, and counting in a liquid scintillation counter. Mean liquid scintillation results for triplicate wells were recorded as counts per minute (cpm). The arithmetic mean of three samples per group was determined \pm the S.D.

IV.C. Results

Construction of plasmids expressing human immunodeficiency virus-like particles

In this study, a non-pathogenic, virus-like particle (VLP) was developed to elicit broad spectrum immune responses to a variety of HIV-1 antigens. The proviral plasmid, pHIV-1_{BH-10}, which encodes for a CXCR4-utilizing virus, was used as a template to construct plasmids expressing VLP. A deletion was introduced into pHIV-1_{BH-10}, by cloning of two PCR products encompassing 1) the regions encoding for *gag-pol* and 2) the *vpu*, *env*, *rev*, and *tat* genes (Figure 7) removing sequences encoding IN, *vif*, *vpr*, and *nef*. In addition, both LTRs were removed. The final plasmid, pVLP_{BH10}, expresses capsid, matrix, nucleocapsid, protease, reverse transcriptase, envelope, Vpu, Tat, and Rev from HIV-1_{BH10}.

The pVLP_{89.6} plasmid was constructed by first making two subclones encoding for the 5' end of the VLP gene insert (*gag-pol* region) and a second subclone encoding for the 3' end of the VLP gene insert (*vpu*, *env*, *tat*, and *rev*). Oligonucleotides were used to amplify each fragment and then subcloned into the expression vector, pTR600. The two fragments were cloned together into a single VLP gene insert (Figure 7). Moreover, four additional 3' PCR products were constructed using sequences from clade B viruses. Each 3' PCR product, encoding the *env* sequences from two R5 HIV-1 isolates, ADA and R2 (a kind gift from Gerald Quinnan), was cloned with the 5' PCR product encoding for the *gag-pol* sequences from HIV-1_{BH10}. Lastly, safety mutations were introduced in the VLP DNA using site-directed mutagenesis to increase the vaccine safety for potential use in humans. These mutations were designed to disable the encapsidation of viral RNA (C15S)³⁹⁵, abrogate RNase H activity (D185N, W266T, E478Q)^{95, 368, 448, 707} and

inactivate the viral protease (D25N) ²⁵.

Additional VLP vaccine constructs were engineered to contain codon optimized Env sequence. pVLP_{coADA}, pVLP_{coJR-FL} and pVLP_{coR2} were generated to express Env from codon optimized sequences from the ADA, JR-FL and R2 strains of HIV-1, while all other proteins were the same as the pVLP_{ADA}. The VLP vaccines were further modified to remove the RT gene (pVLP_{R2/ΔRT}) or add 2 copies of the MPMV CTE (pVLP_{ADA/2xCTE}).

Two SHIV VLP vaccine plasmids were also from made from HIV VLP DNA. pVLP_{SHIV/ADA} contains the sequences for *gag*, PR, RT (SIV_{mac239}), *env*, *vpu*, *tat*, *rev* (SIV_{mac239}). pVLP_{SHIV/ADA/ΔRT} is the same as pVLP_{SHIV/ADA} but does not contain the sequence for RT.

phGag and phGag-Pol (generously donated by Gary Nabel) express core structural and enzymatic proteins from a codon-optimized gene insert ²⁸³. Each of these plasmids encodes for the Gag and Pol gene products using an X4 isolate as the pVLP (Figure 7). Both plasmids were used to compare and contrast to the pVLP and pVLP_{D25N}.

In vitro expression and particle formation of HIV VLPs

Each VLP expressing plasmid was verified for expression in monkey COS cells (Figure 9). Vaccine plasmids expressing VLP with RT and NC safety mutations, but without the protease safety mutations, expressed fully processed Gag and Pol gene products that were detected in both the cell lysates and supernatants of transiently transfected cells (Figures 9A and B). Unprocessed Gag-Pol gene products (Gag-Pol_{p160}, Gag_{p55}, Gag_{p41}) were detected primarily in the cell lysate fraction compared to the

supernatant (Figures 9A and B), whereas Gag_{p24} was detected in both the cell lysates and supernatants. Similar results were observed from cells transfected with phGag-Pol (Figures 9E and F). In contrast, DNA expressing VLP_{D25N} produced incompletely processed Gag-Pol gene products (Gag-Pol_{p160}, Gag_{p55}, Gag_{p41}) (Figures 9C and D). Fully processed Gag_{p24} or Gag_{p17} gene products were not detected in supernatants or cell lysates from cells transiently transfected with DNA expressing VLP with the PR_{D25N} (VLP_{D25N}) mutation. These results were similar to cells transfected with phGag or phGag-Pol_{D25N} (Figures 9E and F). Env was detected in the supernatant of cells (1-2 ng/ml) transfected with DNA expressing VLP and was not affected by the addition of safety mutations. Therefore, each of the DNA plasmids expressing HIV-like particles efficiently expressed all of the structural VLP gene products.

Particles were purified from the supernatants of transiently transfected cells by ultracentrifugation (20-60% sucrose gradient) and collected fractions were analyzed for particle composition and stability. Supernatants from cells transfected with DNA expressing VLP contained particles that banded between 32-40% sucrose (1.14-1.18g/cm³) (Figure 10). These virus-like particles banded in a sucrose gradient similar to wild-type virions. Gag_{p55}, Gag_{p41} and Gag_{p24} were readily detectable in each of these fractions. All VLPs banded in the same percentage of sucrose regardless of whether they were expressed from DNA encoding VLP with or without the NC and RT safety mutations. However, supernatant from cells transfected with DNA expressing VLP with the PR_{D25N} mutation had particles without detectable levels of Gag_{p24}. Similar results were observed from cells transfected with phGag-Pol incorporating PR_{D25N}. However,

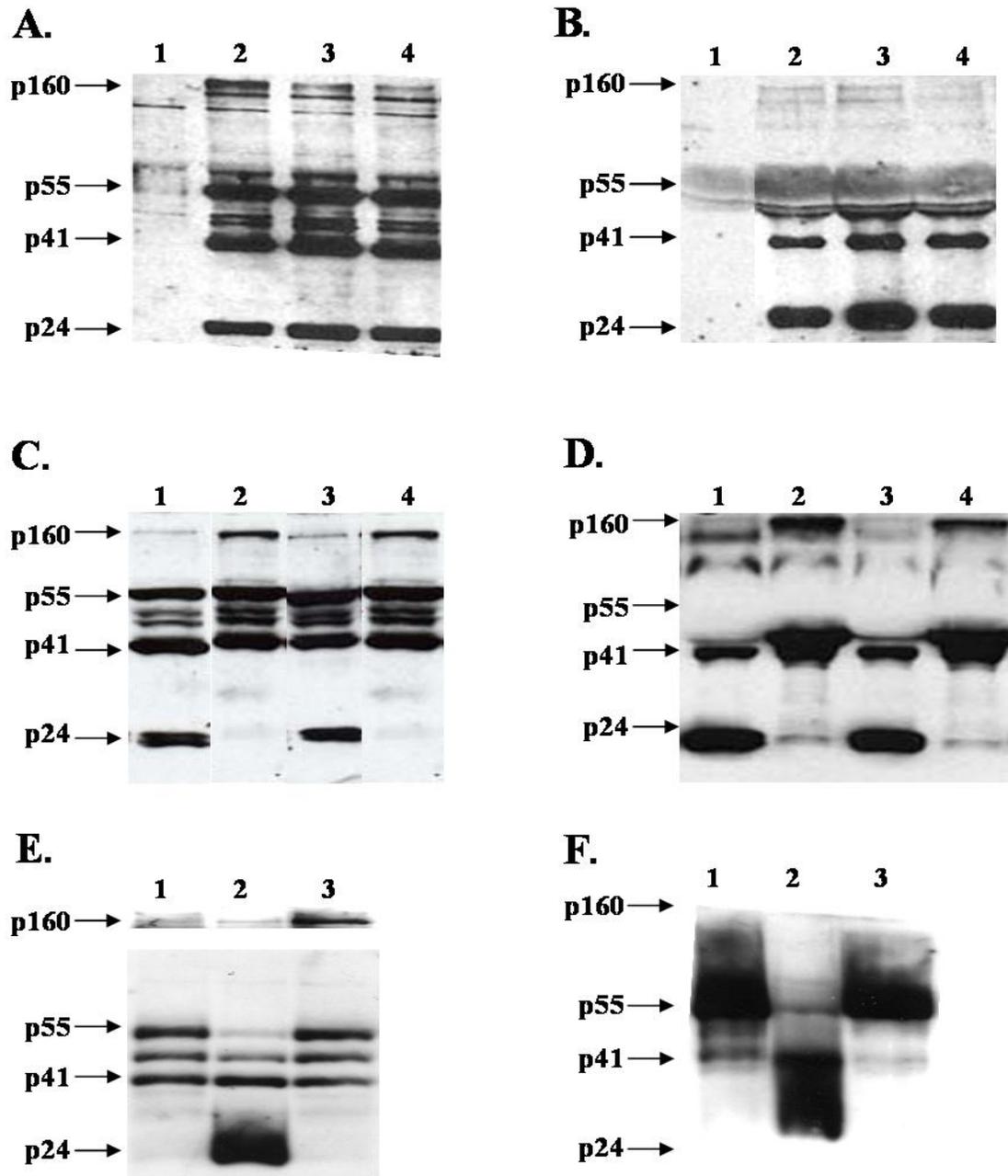


Figure 9. Expression of vaccine constructs *in vitro*.

COS cells were transfected with 2 μ g of each vaccine plasmid. Samples were electrophoresis on a 10% SDS-PAGE (2.0% of cell lysate and 1.5% of supernatant). (A, B) VLP with NC and RT safety mutations; lane 1: pTR600 vector, lane 2: pVLP_{89.6}, lane 3: pVLP_{ADA}, and lane 4: pVLP_{R2}. (C, D) Gag or Gag-Pol VLP; lane 1: pGag_{p55} and lane 2: pGag-Pol_{p160}. (E, F) VLP with NC and RT safety Mutations and protease mutation; lane 1: pVLP_{ADA}, lane 2: pVLP_{ADA(D25N)}, lane 3: pHIV-1_{R2}, and lane 4: pHIV-1_{R2(D25N)}. (A, C, E) cell lysates. (B, D, F) supernatants.

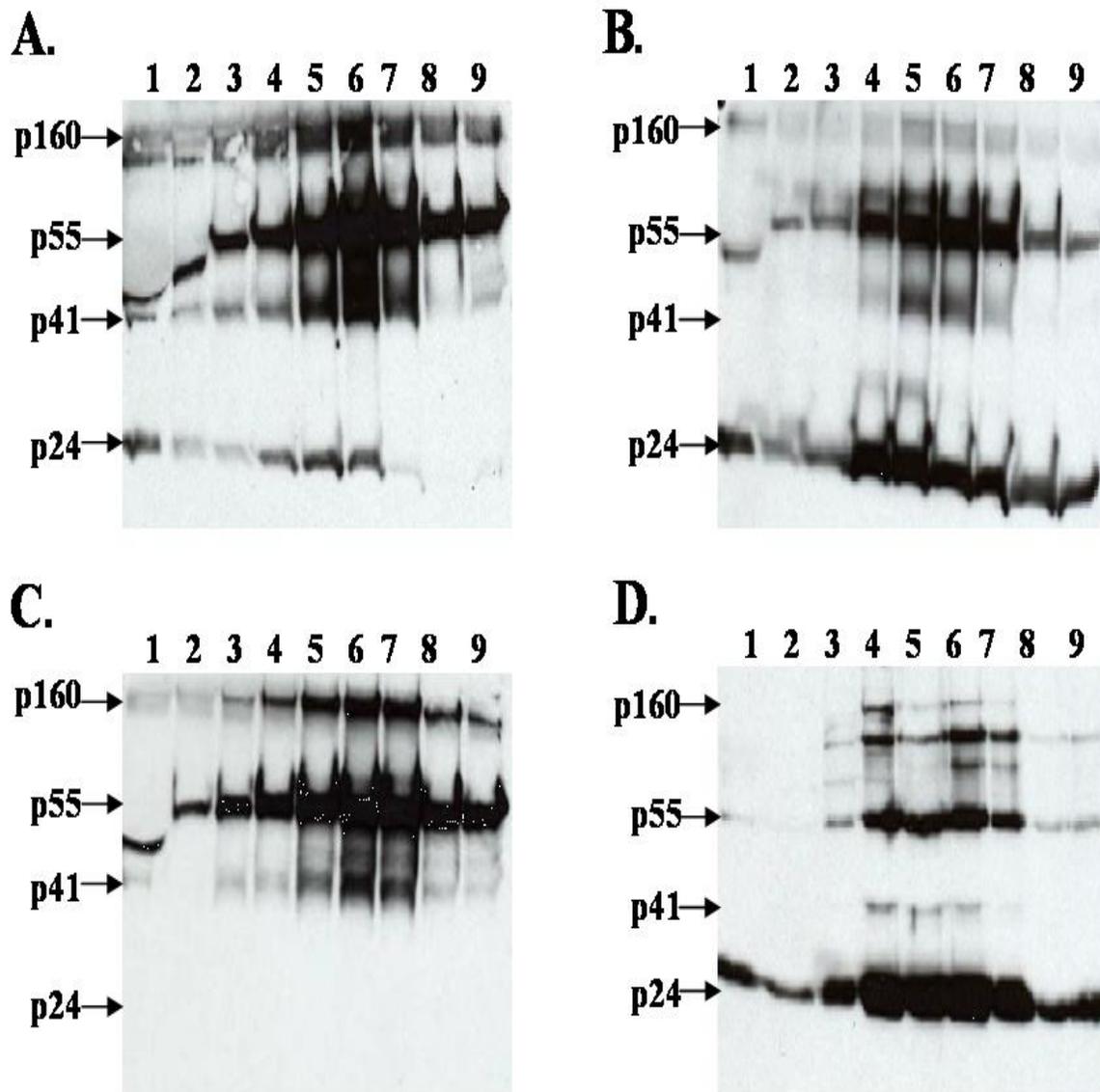


Figure 10. Comparison of sedimentation patterns of secreted viral proteins by sucrose density-equilibrium gradient analysis.

Supernatants from COS cells transiently transfected with plasmid DNA were concentrated through a 20% glycerol cushion and then subjected to 20-60% sucrose density-equilibrium gradient centrifugation. Fractions were collected (ten 1 ml aliquots) from the top of the gradient. Fractions were examined by western analysis. Proteins were probed with HIV-Ig (1:5000) and mouse anti-human IgG (1:7000) and visualized by enhanced chemiluminescence. (A) VLP_{R2}, (B) VLP_{R2(D25N)}, (C) VLP_{R2(G48V)}, (D) HIV-1_{89.6}, (E) Gag_{p55}, (F) Gag-Pol_{p160}.

cells transfected with the phGag-Pol plasmid produced particles that contained predominantly processed Gag gene products with little higher molecular weight Gag-Pol proteins, whereas cells transfected with phGag DNA had unprocessed gene products. Although each DNA plasmid efficiently expressed VLP, the composition of the particles differed depending on PR activity.

VLP vaccines containing codon optimized Env sequences

VLP DNA vaccines containing codon-optimized *env* sequences were constructed to enhance the expression of Env on the virus-like particles. In contrast to VLP DNA with wild-type *env* sequences, VLP vaccines containing codon optimized sequences for Env demonstrated aberrant protein patterns (Figure 10). Each VLP_{coEnv} was transfected into COS cells to test for protein expression and Gag processing. The VLP_{coEnv} constructs had different processing patterns compared to VLPs with wild-type envelopes (Figure 9A/B and 10). All three VLP DNAs with codon optimized *env* sequences expressed viral proteins, but the expression of proteins by pVLP_{coJR-FL} was significantly lower compared to VLP DNA with wild-type envelopes. The introduction of the codon optimized *env* sequence from HIV-1_{JR-FL} into the VLP DNA resulted in minimal protein expression (Figure 10, lane 2). In contrast, the VLP DNA containing the codon optimized *env* sequence from HIV-1_{ADA or R2} expressed more viral proteins compared to pVLP_{coJR-FL} but considerably less compared to VLP DNA with wild-type envelopes. The stark difference in protein expression between VLP DNA with wt or codon-optimized envelopes was demonstrated by analysis of 10 times the amount of sample for VLP DNA with codon-optimized Envs (Figure 10) compared to wt Envs (Figure 9 A and B). In

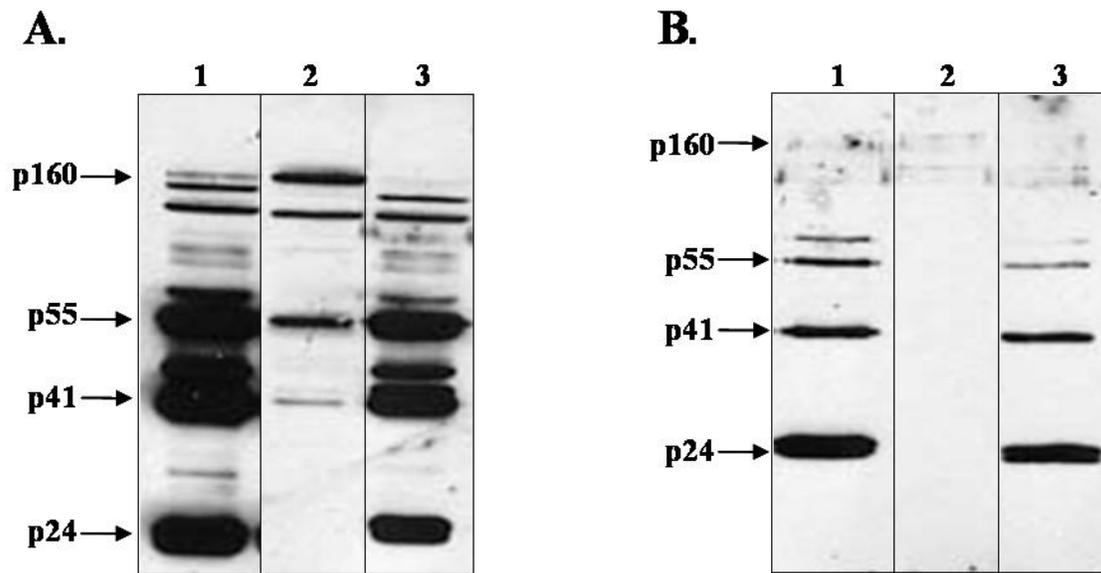


Figure 11. Expression of VLP vaccines containing codon-optimized *env* sequences.

COS cells were transfected with 2 μ g of each vaccine plasmid. Samples were electrophoresis on a 10% SDS polyacrylamide gel. (A) Cell lysates (2.0%) and (B) supernatants (1.5%) were harvested and analyzed for protein expression. Lane 1: pVLP_{coADA}, lane 2: pVLP_{coJR-FL}, and lane 3: pVLP_{coR2}.

addition, the exposure time of the immunoblots to the x-ray film was 15 times longer in the VLP DNA with codon-optimized Envs (Figure 10) compared to wt Envs (Figure 9 A and B). In addition to reduced protein expression, Gag_{p160} was not detected in the cell lysates of cells transfected with pVLP_{coADA} and pVLP_{coR2} (Figure 10, lanes 1 and 2). Therefore, these codon-optimized plasmids were not further characterized due to their aberrant protein expression.

VLP vaccines containing two copies of the MPMV CTE

A VLP DNA vaccine containing two copies of the MPMV CTE was constructed to increase expression of viral proteins and induce VLP production in murine cells. Two copies of the MPMV-CTE were added to the 3' end of pVLP_{ADA}. In murine cells (NIH 3T3), pVLP_{ADA/2xCTE} expresses more viral proteins compared to pVLP_{ADA} (Figure 11, lane 3, 2). Neither of these vaccine constructs produces VLPs in murine cells.

Protein expression of SHIV VLP vaccines

VLP DNA vaccines expressing SIV Gag-Pol and HIV-1 Env, Vpu, Tat and Rev were constructed to test the immunogenicity of VLPs in a non-human primate vaccine/challenge model. SHIV VLP DNA vaccines were analyzed for expression of lentiviral gene products. Transfection of COS cells with pVLP_{SHIV/ADA} and pVLP_{SHIV/ADA/ΔRT} resulted in the expression of proteins that were detected in the cell lysates and supernatants (Figure 13). Similar to the protein expression pattern of pVLP_{ADA} and pVLP_{ADA/ΔRT}, multiple Gag gene products were detected (Gag-Pol_{p160}, Gag_{p55} Gag_{p41} and Gag_{p27}). In addition, the RT-deficient SHIV VLP (pVLP_{SHIV/ADA/ΔRT})

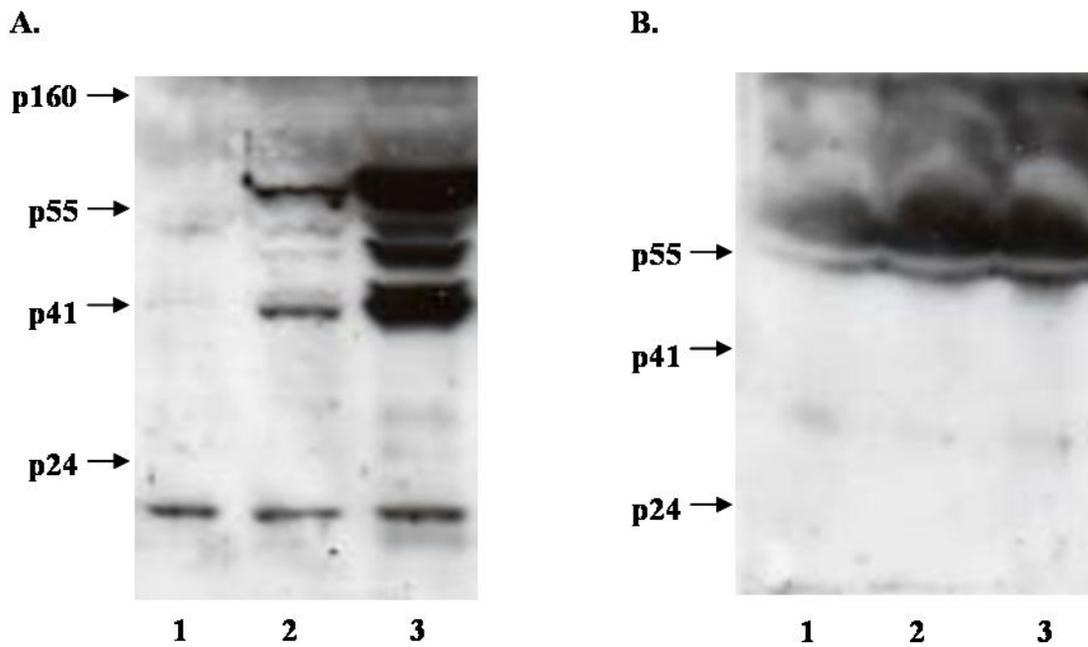


Figure 12. Expression of VLP vaccines in murine cells.

NIH 3T3 cells were transfected with 2 μg of each vaccine plasmid. Samples were electrophoresis on a 10% SDS polyacrylamide gel. (A) Cell lysates (4.0%) and (B) supernatants (5.0%) were harvested and analyzed for protein expression. Lane 1: pTR600, lane 2: pVLP_{ADA}, and lane 3: pVLP_{ADA/2xCTE}.

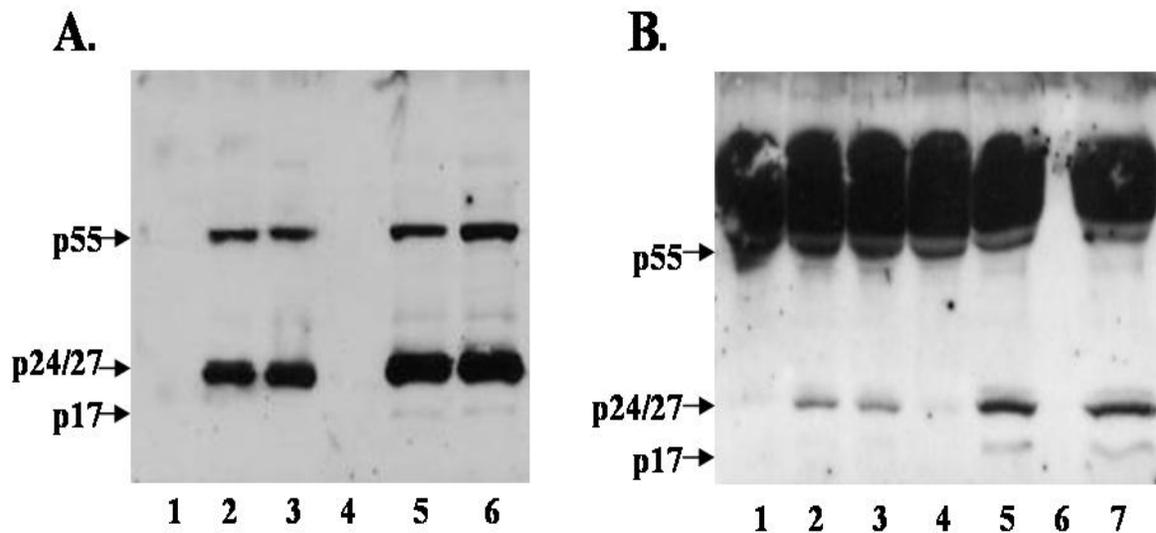


Figure 13. Expression of SHIV VLP vaccine constructs *in vitro*.

COS cells were transfected with 2 μ g of each vaccine plasmid. Samples were electrophoresed on a 10% SDS-PAGE (**A**) 5.0% of cell lysate and (**B**) 5.0% of supernatant) were electrophoresed on a 10% SDS polyacrylamide gel and proteins were detected by western blot analysis (SIV Ig, 1:10000). (**A**) Lane 1: pTR600 vector; lane 2: pVLP_{R2}; lane 3: pVLP_{R2/ΔRT}; lane 4, empty; lane 5: pVLP_{SHIV/ADA}; lane 6, pVLP_{SHIV/ADA/ΔRT}. (**B**) Lane 1: pTR600 vector; lane 2: pVLP_{R2}; lane 3: pVLP_{R2/ΔRT}; lane 4, empty; lane 5: pVLP_{SHIV/ADA}; lane 6, empty; lane 7, pVLP_{SHIV/ADA/ΔRT}. SIV and HIV proteins are indicated on the left.

expressed more proteins compared to SHIV VLP with RT (pVLP_{SHIV/ADA}). These results concur with the protein levels observed with the corresponding HIV VLP vaccines.

Particle formation of HIV and SIV VLP without RT

The sequence encoding for RT was removed in the VLP DNA vaccines to enhance particle production. This smaller VLP insert may also be used in a viral vector system. Particles were purified from the supernatants of transiently transfected cells (pVLP_{R2/ΔRT}, pVLP_{SHIV/ADA} and pVLP_{SHIV/ADA/ΔRT}) by ultracentrifugation (20-60% sucrose gradient) and collected fractions were analyzed for particle composition and stability. Supernatants from cells transfected with DNA expressing VLPs contained particles that banded between 32-40% sucrose (1.14-1.18g/cm³) (Figure 14) similar to pVLP_{R2} (Figure 12A). Gag_{p55}, Gag_{p41} and Gag_{p24/p27} were readily detectable in each of these fractions. Particles produced from pVLP_{R2/ΔRT} and pVLP_{SHIV/ADA/ΔRT} banded in a sucrose gradient similar to wild-type virions (Figure 14), whereas particles were not detected for pVLP_{SHIV/ADA}. Interestingly, the overall amount of Gag gene products was higher for pVLP_{ADA/ΔRT} compared to pVLP_{ADA}.

Accessory protein activity

Rev activity was not measured directly, but was observed as a result of structural HIV-1 antigen expression and particle formation. VLPs secreted into the supernatant of transfected cells indicate that Rev actively promoted the cytoplasmic accumulation of mRNA transcripts encoding for structural gene products¹¹⁹. In addition, DNA expressing infectious virions (pHIV-1_{89.6}) or VLPs (pVLP_{ADA, R2, or 89.6}) had similar levels

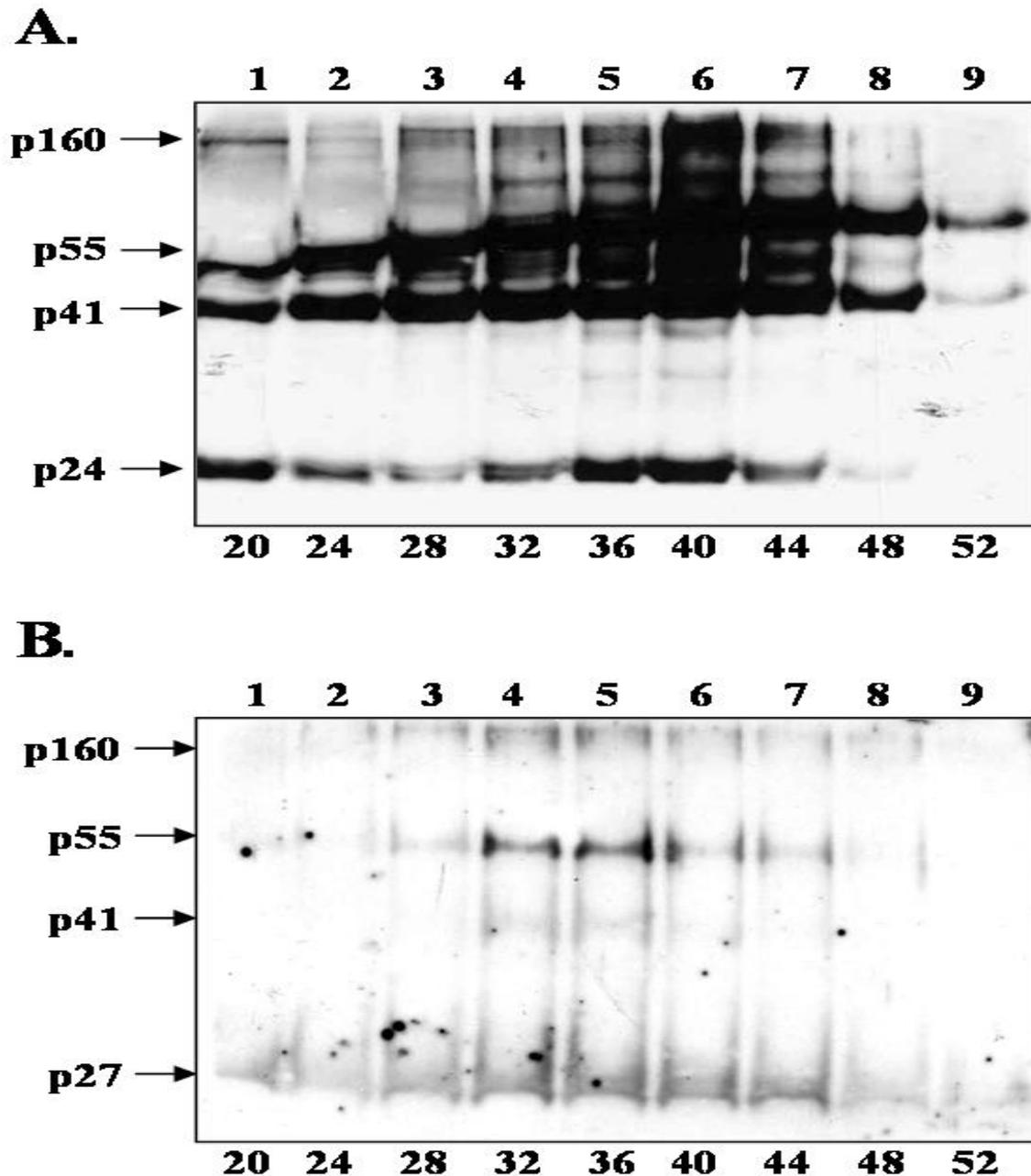


Figure 14. Sedimentation pattern of secreted viral proteins by sucrose density-equilibrium gradient analysis of RT-deficient VLPs.

Supernatants from COS cells transiently transfected with (A) pVLP_{R2/ΔRT} or (B) pVLP_{SHIV/ADA/ΔRT} was concentrated through a 20% glycerol cushion and then subjected to 20-60% sucrose density-equilibrium gradient centrifugation. Fractions were collected (ten 1 ml aliquots) from the top of the gradient. Fractions were examined by western analysis. Proteins were detected with (A) HIV-Ig or (B) SIV Ig (1:10000).

of Tat activity as observed directly using a SEAP reporter assay ¹¹⁸. COS cells were transfected with DNA expressing infectious virions or VLP plus a reporter plasmid containing HIV-1 LTR-SEAP. The level of Tat activation of the LTR was similar regardless of whether Tat was expressed from proviral DNA (pHIV_{ADA}) or DNA encoding VLPs (Table 7). Therefore, DNA encoding VLP transcribed mRNA that efficiently expressed functional Tat and Rev.

Envelope on the surface of HIV VLP binds the human CD4 molecule

VLPs expressed from DNA plasmid bound soluble CD4 (sCD4) (Figure 15). VLP secreted into the supernatant of COS cells were incubated with a soluble form of human CD4. Each VLP, with Env_{89,6}, Env_{ADA}, or Env_{R2} on the surface, bound sCD4 with similar efficiency to wild-type, infectious HIV-1 (Figure 15). Only VLPs containing envelope, and not Gag-only particles, bound sCD4, indicating that the interaction was specific to particles incorporating Env. In addition, cells transfected with a plasmid expressing HIV-1 ADA Env_{gp120} only did not bind to sCD4, because monomeric gp120 was not able to transverse the 20% glycerol layer during purification. Interestingly, even though Env_{R2} on the VLP binds CCR5 independent of CD4, the Env_{R2} on the surface of the VLP bound CD4 with equal efficiency as CD4-dependent envelopes.

Anti-Gag antibodies elicited to the VLP DNA vaccines

The immunogenicity of the DNA expressing VLPs was initially determined in rabbits. New Zealand white rabbits were vaccinated via intramuscular injection with 200 µg of DNA at day 1 and boosted at weeks 6, 12 and 18. Sera from the rabbits immunized

Table 7. Induction of Tat activity.

Plasmid	Relative percentage of Tat activity ^a
pLTR-SEAP	7.0 ± 1.3
pGag-Pol	7.5 ± 0.9
pVLP _{ADA}	100.0 ± 0.0
pVLP _{89.6}	100.0 ± 0.0
pVLP _{R2}	87.0 ± 8.5
pHIV-1 _{89.6}	100.0 ± 0.0

^a Percent of Tat activity relative to pHIV-1_{89.6}.

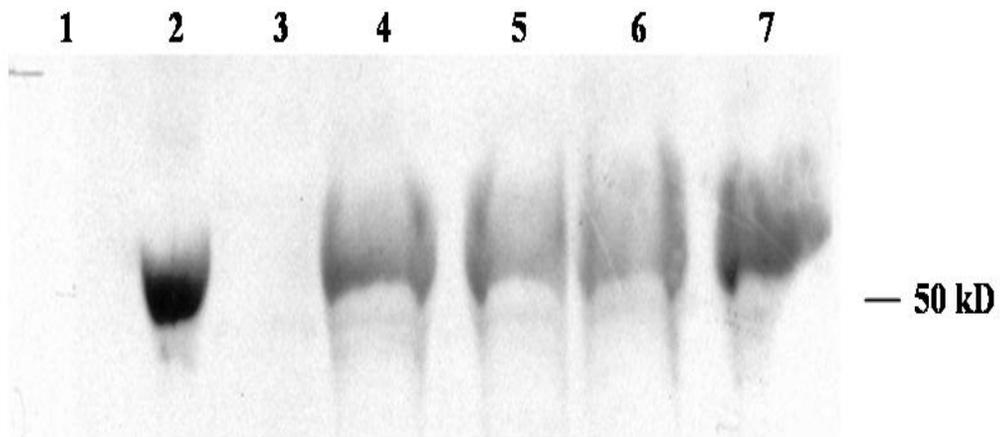


Figure 15. VLP binding to soluble human CD4.

Culture supernatants from COS cells transfected with plasmid DNA were incubated with supernatants from cells expressing sCD4 for 4 h and then pelleted through a 20% sucrose cushion followed by western hybridization analysis. Proteins were probed with rabbit polyclonal anti-hCD4 (1:3000). Antibody bound protein was detected by goat anti-rabbit IgG (1:5000) and visualized by enhanced chemiluminescence. Lane 1: pTR600 vector, lane 2: purified sCD4 control, lane 3: pGag-Pol_{p160}, lane 4: pVLP_{89.6}, lane 5: pVLP_{R2}, lane 6: pVLP_{ADA}, and lane 7: pHIV-1_{89.6}.

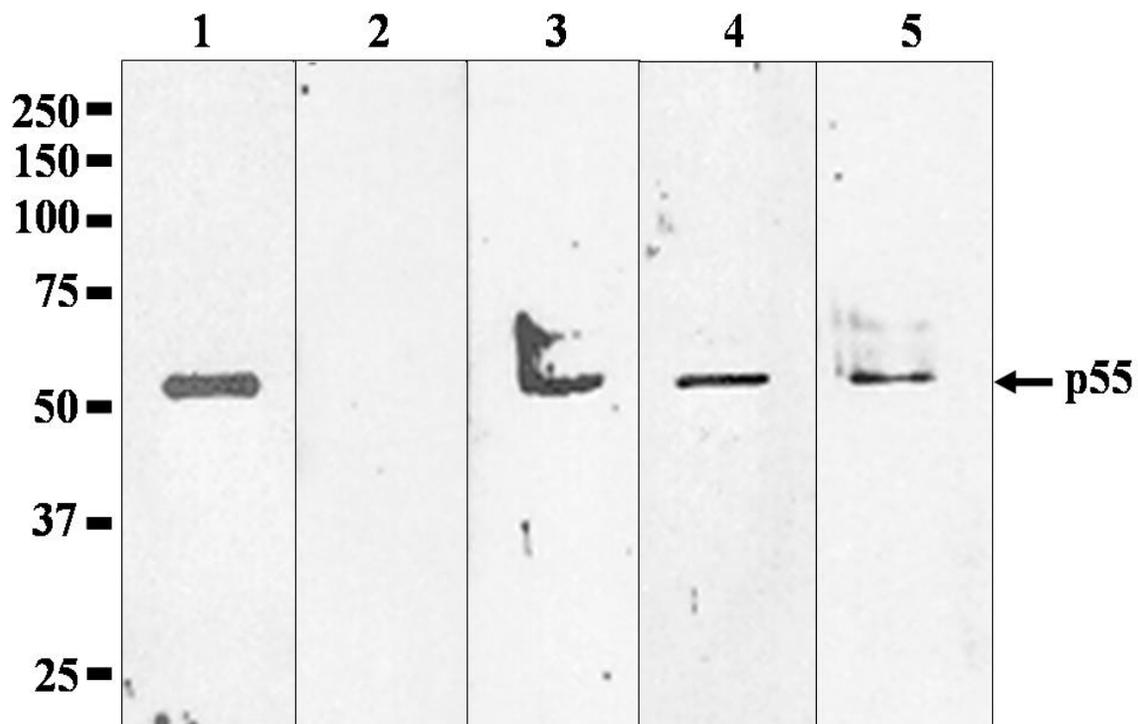


Figure 16. Humoral response to VLP DNA in rabbits.

Sera samples were collected from rabbits two weeks following the last immunization (week 20), and antibody responses were determined by western blot analysis. Purified HIV-1_{SF2} p55 Gag protein was electrophoresed through a SDS polyacrylamide gel and detected by anti-Gag antibodies in HIV-1-infected human (1:3000) or rabbit (1:1500) sera samples. Lane 1, HIV Ig (sera from a patient infected with HIV-1, positive control); lane 2, pTR600; lane 3, pVLP_{89.6} (rabbit #1); lane 4, pVLP_{89.6} (rabbit #2); lane 5, pVLP_{89.6} (rabbit #3).

with the pVLP_{89,6} elicited antibodies that detected HIV-1 antigens (Figure 16). The antisera from three rabbits vaccinated (#1-3) with the pVLP_{89,6} construct contained antibodies that detected Gag_{p55} protein by Western blot analysis, whereas vaccination with pTR600 did induce antibodies that detected Gag_{p55}. A similar approach was performed using purified Env_{gp120}, however the antibody response to Env was below the level of detection.

Anti-Env PBMCs elicited in rabbits vaccinated with VLP DNA

HIV-specific proliferation of PBMCs from vaccinated rabbits was performed to demonstrate the elicitation of anti-Env responses. Rabbits were maintained for an additional 25 weeks after the last immunization and were assayed for anti-Env T-cell responses (Figure 17). Isolated peripheral blood mononuclear cells from the vaccinated rabbits were re-stimulated *in vitro* with purified Env proteins. None of the rabbits contained T-cells that proliferated in response to re-stimulation with either no antigen (mock) or the irrelevant protein (Measles F protein). In contrast, the rabbits vaccinated with pVLP_{89,6} contained PBMCs that proliferated in response to re-stimulation with both HIV_{LAI} and HIV-1_{Ba-L} Env_{gp120} proteins, whereas, the rabbit that received pTR600 did not proliferate in response to the HIV antigens.

IV.D. Discussion

In this study, molecular clones were constructed to express human immunodeficiency virus-like particles as potential vaccine candidates. The composition

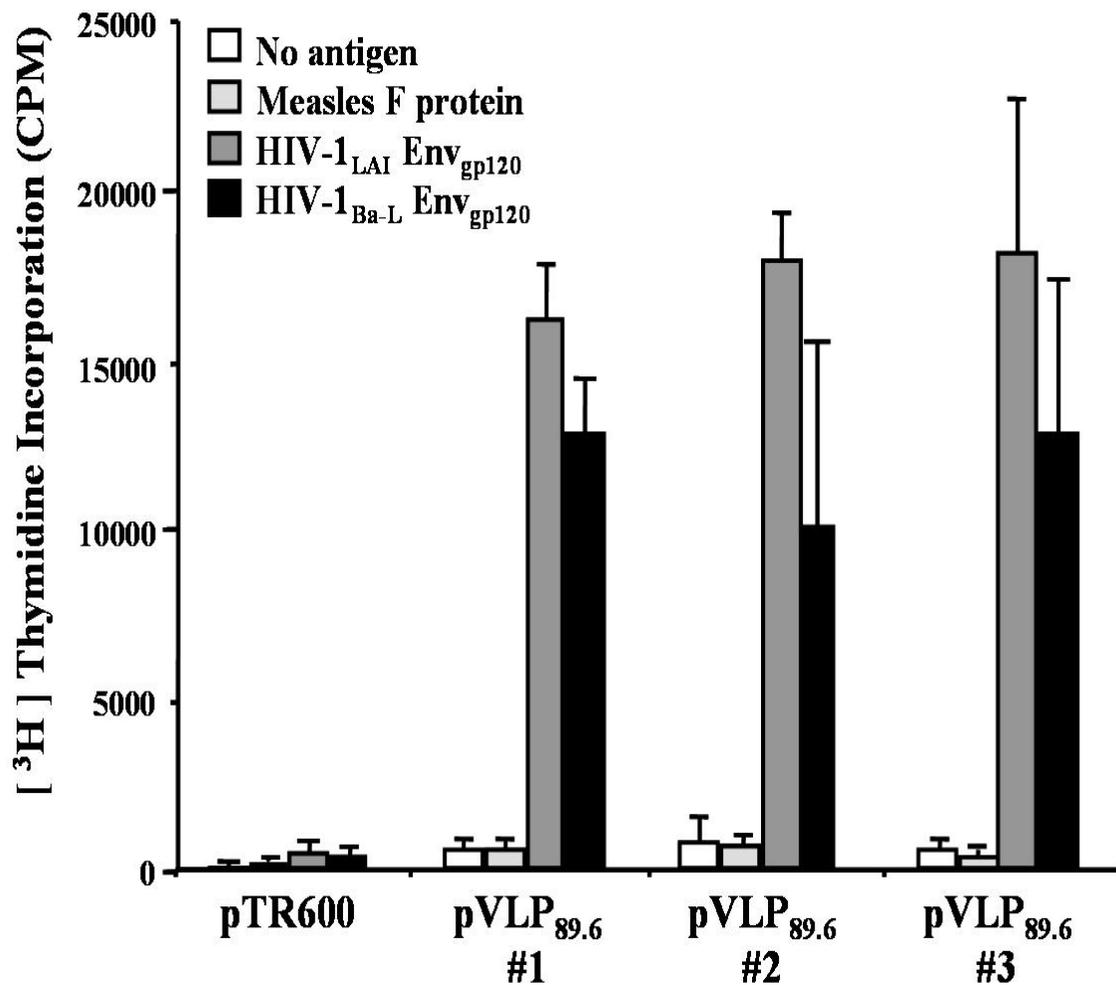


Figure 17. HIV-1 Env-specific proliferation of PBMCs in rabbits vaccinated with VLP DNA.

Rabbits were vaccinated with pTR600 or pVLP_{89.6} (3 rabbits, #1-3) and assayed for anti-Env T-cell responses at week 43 (25 weeks following the last boost). PBMCs were isolated from rabbits and re-stimulated *in vitro* with mock protein, irrelevant protein (Measles F protein) or HIV-1_{LAI} or BaL Env_{gp120} and pulsed with [³H]-thymidine (in triplicate). The incorporation of [³H]-thymidine was given are the arithmetic mean ± the standard deviation (S.D.) for each rabbit.

of the expressed particle has properties that distinguish it from the many VLP for HIV-1 currently in preclinical trials^{71, 319, 389, 464, 719}. In several cases, HIV, as well as SIV, proteins were expressed from more than one plasmid, or the expressed particles were purified and used as the inoculum^{82, 307, 715}. Lentiviral particles, expressed from a single DNA plasmid or from MVA and inoculated into non-human primates, elicited a broad immune response to the expressed antigens^{14, 16}. The virus-like particles expressed from plasmids described in this report can be purified and used as an inoculum or the VLP can be expressed from DNA plasmid or viral vectors *in vivo*. The genome of the VLP contains deletions encompassing the IN, *vif*, *vpr*, and *nef* genes, as well as the 5' and 3' long terminal repeats of HIV-1 (Figure 7). The VLP was encoded by the HIV-1 *gag*, *pol*, *vpu*, *env*, *rev*, and *tat* gene sequences. The deletion of integrase and each viral LTR inhibits the ability of any potential VLP or proviral DNA from inserting into host cell chromosomes¹⁸⁸. The accessory genes (*vpr*, *vif*, and *nef*) have immunomodulatory effects on the immune system, which may be deleterious for HIV vaccine development and therefore were deleted. VLP DNA efficiently expressed the structural gene products (Figure 9) and secreted particles into the supernatant of transiently transfected cells (Figure 12). The regulatory proteins (Tat and Rev) were efficiently expressed and had similar levels of activity as proteins expressed from proviral DNA (Table 1).

VLP vaccines were constructed that contained a substitution in the region encoding for *env*. DNA gene segments encoding for *env*, as well as *tat*, *rev*, and *vpu*, were cloned into the VLP DNA backbone (Figure 7). The *env* gene segments were cloned from one R5X4 (89.6) and two R5 isolates (ADA and R2). The ADA, along with the original 89.6 *env* genes, were selected for two reasons: 1) each envelope is currently

being used by several research groups for HIV vaccine development and therefore would be useful for comparison of elicited immune responses, and 2) X4 envelopes, such as the Env derived from the HIV-1_{BH10} or HIV-1_{IIIB} isolates, are more sensitive to neutralizing antibody compared to primary R5 isolates^{116, 437}. The Env_{ADA} is a particularly difficult envelope to neutralize, and therefore vaccine strategies that elicit neutralizing antibodies using VLP expressing this envelope may elicit a broader immune response to a diverse set of HIV-1 isolates. Env_{R2}, cloned from a patient isolate (HNS2), can be neutralized by sera from patients infected with HIV-1 from clades A, B, C, D, and F, as well as CRF⁵²⁴ and therefore was selected for use as an Env immunogen in the VLP. Virions pseudotyped with the Env_{R2} can mediate CD4-independent infection. In addition, these viruses are sensitive to neutralization by a panel of monoclonal antibodies that recognize conformational epitopes in envelope^{725, 727}. Recently, Dong *et al.* demonstrated that the Env_{R2}, expressed from a VEE replicon, elicited high titer neutralizing antibodies¹⁵¹. Therefore, the 89.6, ADA, or R2 envelopes, incorporated on the surface of the VLP, each has advantages for enhancing the effectiveness of the VLP immunogen.

Second and third generation VLP vaccines were also constructed. VLP DNA vaccines containing codon-optimized sequences were produced to increase the expression of Env on the VLP surface. However, codon-optimization of *env* sequences resulted in aberrant protein patterns (Figure 10). The abnormal protein expression is most likely due to modification of splicing sites. Therefore, the VLP vaccines containing codon-optimized *env* sequences were not further characterized. Next, a VLP DNA vaccine containing two copies of the MPMV CTE (2X CTE) was constructed to increase expression of viral proteins and induce VLP production in murine cells. Under normal

circumstances wild-type HIV-1 or VLP DNA do not produce particles from infected/transfected murine cells. Rabbits vaccinated with the VLP DNA elicited minimal responses to HIV antigens. No anti-Env antibodies and minimal anti-Gag antibodies were detected in the sera of pVLP_{89,6}-vaccinated rabbits. However, Env-specific proliferation of PBMCs from vaccinated rabbits was detected 25 weeks following the last vaccination. In the vaccinated rabbits, the VLP DNA vaccine expressed viral proteins at low levels, but these proteins most likely did not assemble into particles. So, a VLP DNA vaccine containing two copies of the MPMV CTE (2X CTE) was constructed to increase expression of viral proteins and induce VLP production in rodents/small animals. The addition of 2X CTE to the HIV VLP resulted in an increase in protein expression in murine cells (Figure 11). However, particles were still not detected from cells transfected with the HIV VLP with 2X CTE. As an alternative, four tandem copies of the MPMV CTE may overcome the block in assembly and allow VLPs to be produced from murine cells. This approach is currently under investigation. Also, SHIV VLP vaccines have been constructed to examine the immunogenicity and induction of protection of non-human primates from viral challenge. The SHIV VLP vaccines express SIV_{mac239} Gag-PR-RT and Vpu, Env, Rev, and Tat from HIV-1_{R2}. Viral proteins were expressed from cells transfected with SHIV VLP DNA at levels comparable to the HIV VLP DNA (Figure 13). Lastly, HIV and SHIV VLPs were constructed that had the sequence encoding for RT removed to enhance particle production. Viral proteins were expressed and particles budded from cells transfected with HIV or SHIV VLP without RT (Figure 13 and 14). These vaccines expressed viral proteins and produced particles similar to the VLP vaccine containing RT (Figure 12A).

Characterization of these SHIV VLP vaccines is still ongoing. Unlike the VLP vaccines with RT, the RT-deficient VLP insert may be used in a viral vector system. This modification allows the VLP immunogen to become more versatile, therefore allowing the VLP vaccine to be immunized in multiple forms or in a combination (DNA, viral vector, purified particles).

One advantage of incorporating viral envelope glycoproteins on the surface of the VLP is the presentation of Env in a native conformation. The inoculation of monomeric Env_{gp120} elicits high titer anti-Env antibody that does not prevent viral entry into susceptible cells. Various approaches to construct soluble trimeric forms of Env that more closely mimic the native Env_{gp160} on the surface of virions have been employed. Soluble trimerized Env_{gp140}, unstabilized or stabilized with domains (GCN4 or the T4 bacteriophage fibritin motifs or by disulfide linkers)^{1, 51, 60, 563, 578, 710, 711} elicited modest levels of enhancement of neutralizing antibody compared to antibody elicited by monomeric forms of Env. However, even though many of these approaches use a trimerized Env form, there are still distinct differences between soluble, trimerized Env and the structure of the native trimeric form of Env anchored on the surface of the virion. Therefore, the expression of Env in the context of a particle may prove advantageous for vaccine development.

The potential of the VLP RNA to recombine with an HIV-1 genome in an infected patient could limit the use of this vaccine in humans. Therefore, a third generation of VLP expressing plasmids was constructed that incorporated a variety of mutations to increase the safety of these vaccines for potential use in humans. The VLP was modified to prevent 1) the packaging of RNA, 2) reverse transcriptase activity, and

3) the proteolytic processing of Gag-Pol polypeptides. The first set of mutations, introduced into the NC coding region, disrupted the two zinc finger motifs⁴⁴⁹. The principal function of the NC involves the specific encapsidation of full length, unspliced (genomic) RNA into virions. Introduction of serine residues at position 15 and 36 in the NC prevents the binding of zinc and disrupted zinc finger formation in the NC. Previous studies have demonstrated severely restricted packaging of genomic RNA into both infectious virus as well as VLP incorporating these NC mutations^{9, 173, 449}.

During the process of reverse transcription, the viral RNA is converted into proviral DNA in a series of defined steps^{3, 193}. RT activity could have been eliminated by the deletion of the gene sequences encoding for RT. However, at least seven epitopes in RT are immunogenic and are advantageous for eliciting anti-viral immunity²⁸³. The introduction of recombinant HIV-1 RT elicited high-titer CTL responses. Therefore, we chose to disrupt RT activity without preventing the expression of this protein¹⁷³ and thus the presentation of RT epitopes to the immune system.

The final mutation substituted an asparagine for aspartic acid residue at position 25 in protease. Previous studies showed that cells transfected with DNA expressing lentiviral VLP with a mutation at amino acid 25 in PR expressed a precursor molecule with high levels of unprocessed Gag proteins^{173, 502, 602}. Nonetheless, this precursor molecule can be incorporated into a particle through its interactions with the capsid domain of Gag²⁸². The viral protease, similar to other aspartyl proteases, such as renin and pepsin, contains the conserved asparagine-threonine-glycine sequence (protease amino acid positions 25-27) in the active site. The initial critical step for autoprocessing of the Gag-Pol precursor protein is the folding and dimerization of this protein leading to

the formation of the active site. A substitution of an asparagine residue at this position prevents this folding and therefore results in loss of enzymatic activity without an effect on protein structure, which allows for an accumulation of unprocessed Gag_{p55} and Gag-Pol_{p160} in the cell lysate and supernatants (Figure 12). In addition, live virus particles that are expressed from proviral DNA and contain the PR_{D25N} are non-infectious²⁵.

VLP incorporating PR_{D25N} had increased production of unprocessed Gag gene products (Figure 9E and F) and increased numbers of immature particles (Figure 12). The introduction of PR_{D25N} into the VLP has several advantages for vaccine development. VLP with PR_{D25N} reduces the potential for cytotoxicity derived from proteolysis of cellular proteins. In addition, co-expression of proviral DNA and DNA expressing the PR_{D25N} resulted in a dose-dependent decrease in particle maturation and infectivity²⁵. Therefore, expression of these VLP vaccines in an infected patient could have the additional benefit of reducing the infectivity of the wild type virions. Particles secreted from cells transfected with DNA expressing VLP_{D25N} produced predominately unprocessed Gag_{p55} and Gag_{p160} with little processed Gag_{p24} or Gag_{p17} (Figure 12).

Infection with live virus results in virions containing Gag polyproteins that are fully processed during maturation post-budding. These particles are mature and contain a dense capsid core with multiple viral proteins within the particle associated with the genome RNA. In addition, human immunodeficiency virus-like particles are immature, contain mostly processed Gag gene products and have severely reduced levels of VLP RNA packaging¹⁷³. In contrast, VLP_{D25N} is composed of surface envelope on primarily unprocessed Gag-Pol_{p160} and Gag_{p55} (Fig 12B). Each of these various particles may have advantages for eliciting a broad range of immune responses.

These VLPs have distinct advantages for vaccine development. First, the deletion of the viral LTR, *integrase*, *vpr*, *vif*, and *nef* sequences enhances the safety of the vaccine and reduces the immunomodulatory effects of the immunogen. The introduction of mutations or deletions in RT and the NC further increases the safety of this vaccine by reducing the infectivity of these particles. Most live or live-attenuated viruses contain viral RNA and are therefore replication-competent with the ability to produce pathogenesis in vaccinated hosts. The lack of particle-encapsidated RNA and the inability of the vaccine insert to integrate into host chromosomes are advantages of this VLP vaccine. A second advantage of this VLP is the gene products can be expressed from a single DNA plasmid. The ability to characterize one DNA plasmid expressing the entire VLP reduces the potential regulatory and safety concerns associated with testing multiple DNA plasmids for prospective use in humans. Third, VLP_{D25N} produces particles with predominately unprocessed, higher molecular weight Gag gene products that may elicit higher levels of anti-HIV CTL responses compared to particles with fully processed Gag gene products. Recently, it was demonstrated that splenocytes from mice vaccinated with particles composed of Gag_{p55} lysed a higher number of target cells presenting Gag peptides compared to cells from mice vaccinated with particles composed of Gag-Pol_{p160}²⁸³. Fourth, the inactivation of the viral protease may lead to markedly reduced shedding of gp120²⁵⁰. CD4 binding to Env increases shedding of Env_{gp120} from viral particles, and Env_{gp120} may also be shed from the VLP during the purification process⁴³⁵. Thus, VLP_{D25N} has the added advantage of retaining Env and thereby increasing anti-Env immune responses. Lastly, the VLPs described in this report have the added advantage of presenting Env epitopes to the immune system and therefore

eliciting both a broad spectrum antibody and cell-mediated immune response.

An effective vaccine against HIV/AIDS will most likely need to elicit high levels of cross-reactive neutralizing antibodies in combination with a robust cell-mediated response against multiple viral antigens to protect from disease in an infected host. One approach to achieve these goals is the development of a human immunodeficiency virus-like particle. In general, these particle-based strategies present multiple viral proteins in the conformation similar to the live virus without the safety issues associated with replication-competent virions. Current virus-like particle vaccines are similar in nature to live-attenuated viruses, expressing both structural and enzymatic proteins. The virus-like particle vaccine described in this study does not produce a productive infection or integrate into host chromosomes. The non-replicative nature of this type of vaccine prohibits the particles from reverting or recombining with wild-type vRNA to produce a pathogenic form. The incorporation of additional safety mutations enhances the value of these vaccines for use in humans.

IV.E. Acknowledgments

We would like to thank Gary Nabel for providing the plasmids expressing Gag and Gag-Pol gene sequences and Gerald Quinnan for providing the plasmid expressing Env_{R2}. HIV-Ig and recombinant Env_{gp120} were obtained from the National Institute of Health AIDS Research and Reference Reagent Program. We also thank Joseph Bower for critical reading and comments. This research was supported by awards AI49061 and AI51213 from the National Institute of Allergy and Infectious Diseases to T.M.R.

V. Chapter 5: Specific Aim II

**Elicitation of Mucosal and Systemic Immune Responses by a Human
Immunodeficiency Virus-Like Particle Vaccine**

V.A. Introduction

Human immunodeficiency virus type 1 (HIV-1), similar to many other pathogens, is primarily transmitted at the mucosal surface. Yet, the majority of experimental vaccines against HIV-1 have been inoculated parenterally. Although an efficient systemic response develops, immunity at the mucosa after parenteral inoculation is negligible. In contrast, mucosal immunization can induce immune responses at both local and distal mucosal sites as well as systemic immunity (For reviews see references ^{100, 128, 372, 376}). An ideal HIV/AIDS vaccine will most likely need to elicit robust immunity against multiple viral antigens in both systemic and mucosal immune compartments ⁶¹⁰.

The mucosal immune system is comprised of an integrated network of lymphoid cells that interacts with innate host factors to preserve the integrity of the host defense. HIV can infect Langerhans cells located in the pluristratified epithelial layer of the vagina or dendritic cells in the rectal cavity and transverse the intact epithelial barrier ¹⁰⁰. These dendritic cells have high expression of DC-SIGN which promotes the internalization of the virus ^{211, 277, 360, 481, 618, 685}. Consequently, HIV infects a substantial number of CD4+ T cells found in these tissues. Eventually, this localized infection rapidly leads to widespread infection of the host. In contrast to the viral load in the blood, treatment with antiviral drugs does not lower the viral titer in the rectal mucosa ¹⁴⁷. In addition, Langerhans cells in the epithelium and lamina propria in the vagina and rectal mucosa can act as reservoirs of viral infection ^{46, 147, 515}.

Protection against mucosal pathogens is conferred by a combination of antibodies

(IgG and sIgA) and CTLs^{35, 37, 67, 350, 368}. IgG and sIgA block HIV infection of susceptible cells³⁴⁷. These anti-HIV antibodies can prevent viral transcytosis across a mucosal monolayer^{10, 54}, neutralize virus infectivity¹⁴⁶, and mediate antibody-dependent cellular cytotoxicity⁵³. Additionally, containment of viral replication by lysis of infected cells is achieved primarily by CTLs⁴²⁴. The number of antigen-specific cytotoxic CD8+ T cells in the lamina propria of the gut is directly proportional to the level of protection after mucosal viral challenge^{35, 425, 466}. Therefore, augmentation of the immune response at the mucosa by mucosally-administered vaccines induces high titer anti-HIV immunity and is more likely to prevent viral infection and the establishment of viral reservoirs compared to vaccines inoculated parenterally.

A particle-based immunogen, such as a non-infectious virus-like particle (VLP), is a promising candidate for a safe and effective mucosal vaccine against HIV-1^{13, 14, 16, 76, 82, 151, 173, 399, 603, 712, 713, 715, 720}. VLPs or pseudovirions can be defined as self-assembling, non-replicating, non-pathogenic, genomeless particles that are similar in size and conformation to intact infectious virions. There are multiple combinations of viral proteins that may be used to generate VLPs, however, they must contain Gag gene products in order to undergo the assembly and budding process. Env_{gp160} may also be processed and incorporated as trimeric spikes protruding from the surface of the particle. The VLP used in this study is expressed from a multi-gene DNA vaccine. Unlike many other VLPs currently being used in AIDS vaccine research^{44, 76, 82, 92, 283, 341, 376, 379, 471, 640, 672-674, 712, 715}, our multi-gene VLP DNA plasmid expresses Gag, Pol, Env, Vpu, Tat, and Rev gene products intracellularly and secretes VLPs extracellularly from a single gene insert⁷²⁰.

The multi-gene VLP vaccine approach described in this study has several advantages compared to live-attenuated virus or single gene vaccines. These VLPs: 1) do not integrate into host genomes⁷²⁰, 2) express multiple viral epitopes that have the potential to stimulate a diverse set of immune responses, 3) have point mutations introduced into the VLP genome to abrogate reverse transcriptase and RNase H activity^{395, 449}, as well as to restrict viral RNA packaging^{25, 368, 707}, and 4) have the potential to bind and enter cells expressing appropriate receptors such as macrophages and dendritic cells. In addition, cell-free VLPs bound with antibodies can be phagocytized by macrophages and dendritic cells via Fc receptors, thus enhancing MHC class II presentation of viral antigens.

In this study, mice were intranasally vaccinated with purified VLPs alone or co-inoculated with CpG ODNs. Anti-Env antibodies were detected in both the sera and mucosa. In addition, anti-Env and anti-Gag splenocytes were induced in vaccinated mice. Mice co-vaccinated with VLPs plus CpG ODNs had significantly enhanced cellular and humoral responses to HIV antigens. In conclusion, systemic and mucosal immune responses to HIV-1 are enhanced by intranasal immunization of purified virus-like particles expressed from a virally-regulated multi-gene DNA vaccine.

V.B. Material and Methods

DNA Plasmids

The plasmids, pVLP_{ADA} and pVLP_{R2}, express VLPs and have been previously characterized⁷²⁰. Briefly, each VLP is expressed from sequences including: HIV-1_{BH10}

gag-pol (pHIV_{BH10} nt 112-3626) and HIV-1_{ADA or R2} *vpu, env, rev, tat* (pHIV_{BH10} nt 5101-8159). Both the 5' and 3' LTRs have been deleted along with the sequences encoding for IN, Vpu, Vif, and Nef. Mutations were introduced into the *gag* and *pol* genes to inhibit reverse transcriptase activity, RNase H activity, strand transfer, and to restrict viral RNA packaging⁷²⁰. Each VLP is efficiently secreted from primate cells. The plasmid, pVLP_{2xCTE}, is derived from pVLP_{ADA}. Two copies of MPMV CTE and 200bp of nonsense sequence was molecularly cloned into pVLP_{ADA} using unique restriction sites, *NheI* and *SacII*.

Transfections and expression analysis

Monkey (COS) or murine (NIH 3T3) fibroblastic cell lines (5 X 10⁵ cells/transfection), were transfected with DNA (2 µg) using lipofectamine (12%) according to the manufacturer's guidelines (Life Technologies, Grand Island, NY, USA). Supernatants (2 ml) and cell lysates (300 µl dH₂O) were collected and stored at -20°C. For Western blot analysis, samples were diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA, USA), boiled (5 min), and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and incubated with polyclonal human HIV-infected patient antisera (HIV-Ig) (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk). After extensive washing, bound human antibodies were detected with horseradish peroxidase-conjugated goat anti-human antiserum (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk) and enhanced by chemiluminescence (Amersham, Buckinghamshire, UK).

Purification of virus-like particles.

Supernatant from COS cells, transiently transfected with VLP-expressing DNA, were pelleted (2 h at 4°C) via ultracentrifugation (100,000 x g through 20% glycerol, weight per volume). The pellets were subsequently resuspended and used for 1) *in vivo* inoculation or 2) sucrose gradient ultracentrifugation. Pellets used for immunization were resuspended in sterile PBS. Total protein concentration was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). In addition, the level of non-HIV protein contamination (from the media/cells) in each inoculum was visualized by a silver-stained SDS-PAGE using the ProteoSilver Silver Stain Kit (Sigma, St. Louis, MO, USA). The specificity of each inoculum was analyzed in a separate immunoblot with HIV Ig as described earlier.

Pellets were analyzed for particle formation by resuspending the material in PBS and overlaid onto 20-60% sucrose gradients (11 steps, 4% increments) and ultracentrifuged (17 h at 100,000 x g at 4°C). Eleven fractions (20-60% sucrose, 1 ml, weight per volume in PBS) were collected (top to bottom) from the gradient, and the proteins were precipitated with equal volumes of 20% TCA and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by HIV Ig via western blot and visualized by enhanced chemiluminescence. In addition, the purity of each VLP preparation in each inoculum was visualized by a silver-stained polyacrylamide SDS gel.

Immunization of mice

Female BALB/c mice (5–7 weeks old) were immunized at weeks 0, 3, and 6 with purified VLPs (40 µg total protein) alone or co-inoculated with phosphorothioate CpG

oligodeoxynucleotides (CpG ODNs, 10 µg each) via the nares. Each CpG ODN (ODN-1: 5'-TCCATGACGTTTCCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3')^{203, 274, 307, 308, 428, 451} was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile dH₂O (2µg/ul) and stored at -80°C. Mice were administered VLPs in sterile PBS +/- CpG ODNs into the nares of each mouse (40 µl total volume). Supernatants from primate cells transiently transfected with the vector, pTR600, were ultracentrifuged through 20% glycerol. The pellet was resuspended in sterile PBS +/- CpG ODNs and inoculated into the naïve mice (equivalent volume compared to mice given a VLP inoculum). Mice were housed in compliance with U.S.D.A. regulations and were monitored daily for weight loss, behavior, and adverse reaction. Mice were partially anesthetized with xylazine (20 mg/ml) and ketamine (100 mg/ml) administered subcutaneously in the abdomen prior to immunization.

Collection of samples

Blood samples were collected by retro-orbital plexus puncture on day 1 and weeks 5 and 8 post-immunization on anesthetized mice. Sera samples were collected by centrifugation (5,000 rpm, 10 mins) and stored at -80°C. Collected sera were stored in a -20°C freezer. Mucosal washings were collected at week 9 post-immunization. Vaginal lavages were collected by repeated rinsing of the vagina (200 µl) with sterile PBS. The lungs were excised and cut into small pieces using a sterile scalpel in sterile PBS (200 µl). The lung tissue pieces were centrifuged (12,000 rpm, 5 min), and supernatants were

collected. A section of the intestines (1.3 cm) was aseptically removed and placed in sterile PBS (200 μ l). The collected fecal samples were disrupted using a sterile pipette tip, vortexed (15 sec), centrifuged (12,000 rpm for 5 min), and the product supernatants were collected. The protein concentration of the fecal supernatants was adjusted to a final concentration of 1 mg/ml with sterile PBS. All samples were stored at -80°C.

Antibody responses to VLP immunizations

Serum and mucosal samples were individually collected and tested for antibody responses to Env by ELISA. Supernatants collected from 293T cells transiently transfected with a plasmid that expressed Env_{gp120} from either HIV-1_{ADA} (pcoEnv_{ADA}) or HIV-1_{R2} (pcoEnv_{R2}). Each well of a 96-well plate was coated with culture supernatant (50 ng Env_{gp120} per well) (4°C for 16 hr). Plates were blocked (25°C for 2 hr) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%) and then incubated with serial dilutions of each sample (sera or mucosal washings) (25°C for 2 hr). Following thorough washing in PBS-Tween 20 (0.05%), samples were incubated (25°C for 1 hr) with biotinylated goat anti-mouse IgG (1:5,000) diluted in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Streptavidin-HRP (1:7,000) was diluted in PBS-Tween 20 (0.05%) and incubated (25°C for 1 hr). Samples were incubated with TMB substrate (1 hr), and the colorimetric change was measured as the O.D. (450nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The O.D. value of naïve sera and sera from mice vaccinated with virus-like particles composed of only Gag gene products (no Env) was subtracted from the samples using antisera from vaccinated mice. Results were recorded as the arithmetic

mean \pm the S.D. The endpoint titer was expressed as the inverse of the dilution of the well that was equal to the well that did not receive serum sample. In order to measure the level of HIV-1 Env-specific IgG subtypes, biotinylated goat anti-mouse IgG₁ or IgG_{2a} (1:5,000) antibodies were used to detect Env-antibody complexes. Results are given as the ratio of the endpoint titers of IgG_{2a} to IgG₁.

Antisera from vaccinated mice were tested for the ability to neutralize virus infection *in vitro* using TZM-B1 cells indicator cells⁴⁵². These cells express human CD4 (hCD4), human CCR5 (hCCR5) and human CXCR4 (hCXCR4) and a luciferase reporter driven by the HIV-1 LTR. TZM-B1 cells were cultured in cDMEM. Infectivity was determined using serial dilutions of antisera with cells in complete, non-selective media in the presence of DEAE dextran (20 μ g/ml) (25°C for 1hr). Cells 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) (48 h) and then clarified by centrifugation. The lysates were mixed with luciferase substrate (2 mg/ml) (20 μ l each). Virus neutralization by mouse antisera was determined in triplicate by measuring the RLU using a Femtomaster FB12 Luminometer (Zylux, Maryville, TN). Neutralization by naïve sera and sera from mice vaccinated with virus-like particles composed of only Gag gene products (no Env) was subtracted from the RLU from assays using antisera from vaccinated mice.

Cell-mediated immune responses to VLP antigens

Spleens were harvested from vaccinated mice at week 9, and splenocytes were isolated. Splenocytes were depleted of erythrocytes by treatment with ammonium

chloride (0.1 M, pH 7.4). Following thorough washing with PBS, cells were resuspended in RPMI medium with 10% fetal bovine serum (cRPMI). Cell viability was determined by trypan blue exclusion staining. The number of anti-Gag_{p24} or anti-Env_{gp160} specific murine INF- γ (mINF- γ) secreting splenocytes was determined by ELISPOT assay (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-mINF- γ plates were incubated (25°C for 2 hr) with cRPMI (200 μ l) and then were incubated with splenocytes (1 X 10⁶/well) isolated from vaccinated mice. Splenocytes were stimulated (48 hrs) with peptides representing the HIV-1 Gag_{p24} (HIV-1 consensus B Gag: GHQAAMQMLKETINE and AMQMLKETINEEAAE) or Env_{gp160} (NA consensus: KSIHIGPGRAFYYTTG and B-subtype isolate: KSIPMGPGKAFYATG). Additional wells of splenocytes were stimulated with PMA (50 ng)/ionomycin (500 ng) or were mock stimulated. In addition, IL-2 was added to all wells (10 units/ml). Plates were washed with PBS-Tween (3X) and were incubated (25°C for 2 hr) with biotinylated anti-mINF- γ and incubated (4°C for 16 h). The plates were washed and incubated (25°C for 2 hr) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1 hr) with stable BCIP/NBT chromagen. The plates were rinsed with dH₂O and air dried (25°C for 2 hr). Spots were counted by an ImmunoSpot reader (Cellular Technology, Cleveland, OH, USA).

V.C. Results

Construction of vaccine plasmids

Each VLP vaccine plasmid has been constructed and characterized in Chapter IV⁷²⁰. Briefly, each plasmid encodes for the Gag and Pol gene products using sequences from HIV-1_{BH10} and Env, Vpu, Tat, and Rev from sequences isolated from two strains, HIV-1_{ADA} or HIV-1_{R2} (Figure 7). Sequences encoding IN, Vif, Vpr, and Nef were deleted, as well as sequences representing the LTRs. Lastly, safety mutations were introduced in the VLP genome to abrogate the encapsidation of viral RNA³⁹⁵ and RNase H and RT activity^{95, 368, 448, 707}.

In vitro expression and particle purification of virus-like particles

Expression of virus-like particles from each DNA plasmid was verified in primate (COS) or rodent (NIH 3T3) cell lines (Figure 18A). Gag and Pol gene products were detected in both the supernatant and cell lysates from transiently transfected primate cells. Murine cells transfected with VLP expressing DNA had Gag and Pol gene products in the cell lysate fraction, but no HIV-1 proteins were detected in the cytoplasm consistent with expected block in HIV-1 assembly in murine cells. Particles were purified from the supernatants of transiently transfected COS cells by ultracentrifugation (20-60% sucrose gradient), and collected fractions were analyzed for particle composition and stability. VLPs banded in sucrose fractions (32-40% sucrose, 1.14-1.18g/cm³) (Figure 18B), in the same fractions as previously described for wild-type virions^{145, 671}.

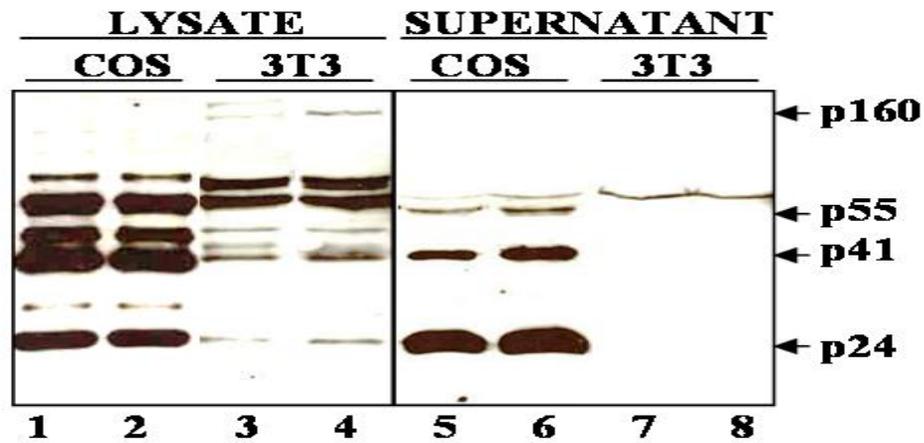
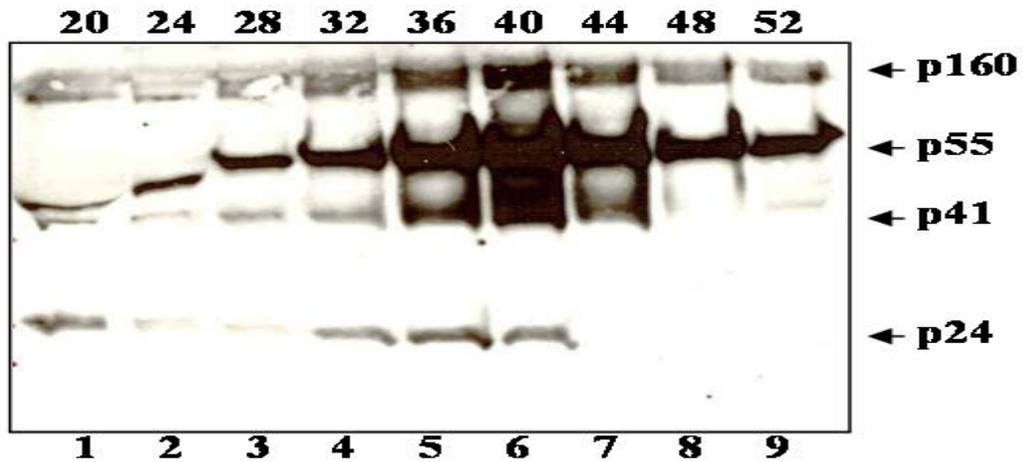
A.**B.**

Figure 18. Expression of viral proteins in primate and rodent cells.

(A) Protein expression of VLP DNA. COS and NIH 3T3 cells were transfected with 2ug of each vaccine plasmid and protein expression was analyzed by western blot analysis. Lanes 1, 3, 5, and 7: pVLP_{ADA} and lanes 2, 4, 6, and 8: pVLP_{R2}. Lanes 1-4: 3.3% cell lysates and lanes 5-8: 1.5% of supernatants. (COS, monkey fibroblasts and 3T3, NIH 3T3 murine fibroblasts; LYS, cell lysate and SUP, supernatant) (B) Virus-like particle production by sucrose density gradient ultracentrifugation. Supernatants from COS cells transiently transfected with VLP_{R2} DNA were concentrated through a 20% glycerol cushion and then subjected to 20-60% (w/v) sucrose density gradient-equilibrium gradient ultracentrifugation. Fractions were collected from top to bottom, precipitated with 10% TCA, and proteins were detected by western blot analysis. Percent of sucrose fraction is located at the top of each lane.

Effectiveness of particle purification process

Samples from each step during the purification process were analyzed for the non-HIV proteins (Figure 19). Samples from different time points were electrophoresed on a 10% SDS polyacrylamide gel and silver stained. Supernatants from COS cells transfected with pTR600 contained an enormous amount of non-HIV protein following the clarification process (centrifugation and 0.22 μm filter) (0.1% of sample) (lane 3). After supernatants were concentrated through a 20% glycerol cushion, there was a precipitous drop in non-HIV proteins detected (>99% non-HIV proteins in supernatant did not transverse the 20% glycerol) (100% of sample) (lane 4 verses lane 3). After the samples were subjected to 20-60% sucrose density-equilibrium gradient ultracentrifugation, non-HIV proteins were below the level of detection (100% of fractions 32-44%) (lane 5).

Elicitation of anti-Env antibody responses

Mice (BALB/c) were vaccinated intranasally with purified VLPs (20 μg of total protein per nare) alone or co-immunized with CpG ODNs (10 μg) at day one and weeks 3 and 6. Mice vaccinated with VLP plus CpG ODNs elicited higher serum titers (> 1 log) of anti-Env antibodies than mice vaccinated with VLP alone (Figure 20A), which continued to rise following the third inoculation (week 8). Mice vaccinated with VLP plus CpG ODNs had statistically higher anti-Env titers (1:10,000-50,000) than mice vaccinated with VLP only (1:900-4,500) (Figure 20B). Additional mice were vaccinated with 10 μg of purified, soluble HIV-1_{ADA} trimers (+/- 10 μg CpG ODNs) as a positive control, and the anti-Env endpoint titers were 1:1920 and 1:696320, respectively. There

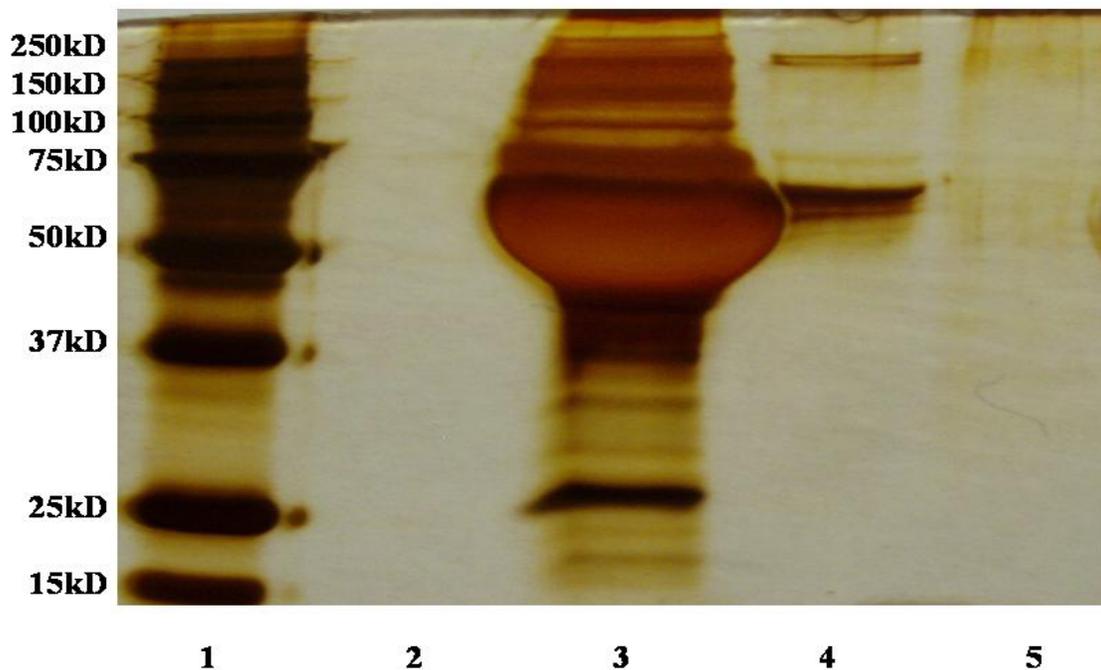


Figure 19. Effectiveness of the particle purification process.

COS cells (T75 flask) were transfected with TR600 (5 μ g). Supernatant (10 ml cDMEM) was concentrated through a 20% glycerol cushion and then subjected to 20-60% sucrose density-equilibrium gradient centrifugation. Fractions were collected (ten 1 ml aliquots) from the top of the gradient. Samples from different timepoints during the purification process were electrophoresed on a 10% SDS polyacrylamide gel and silver stained. Lane 1: protein marker; lane 2, empty; lane 3, 0.1% of supernatant from cells transfected with pTR600; lane 4: 100% of 20% glycerol pellet; lane 5, 100% of 32-44% sucrose fractions.

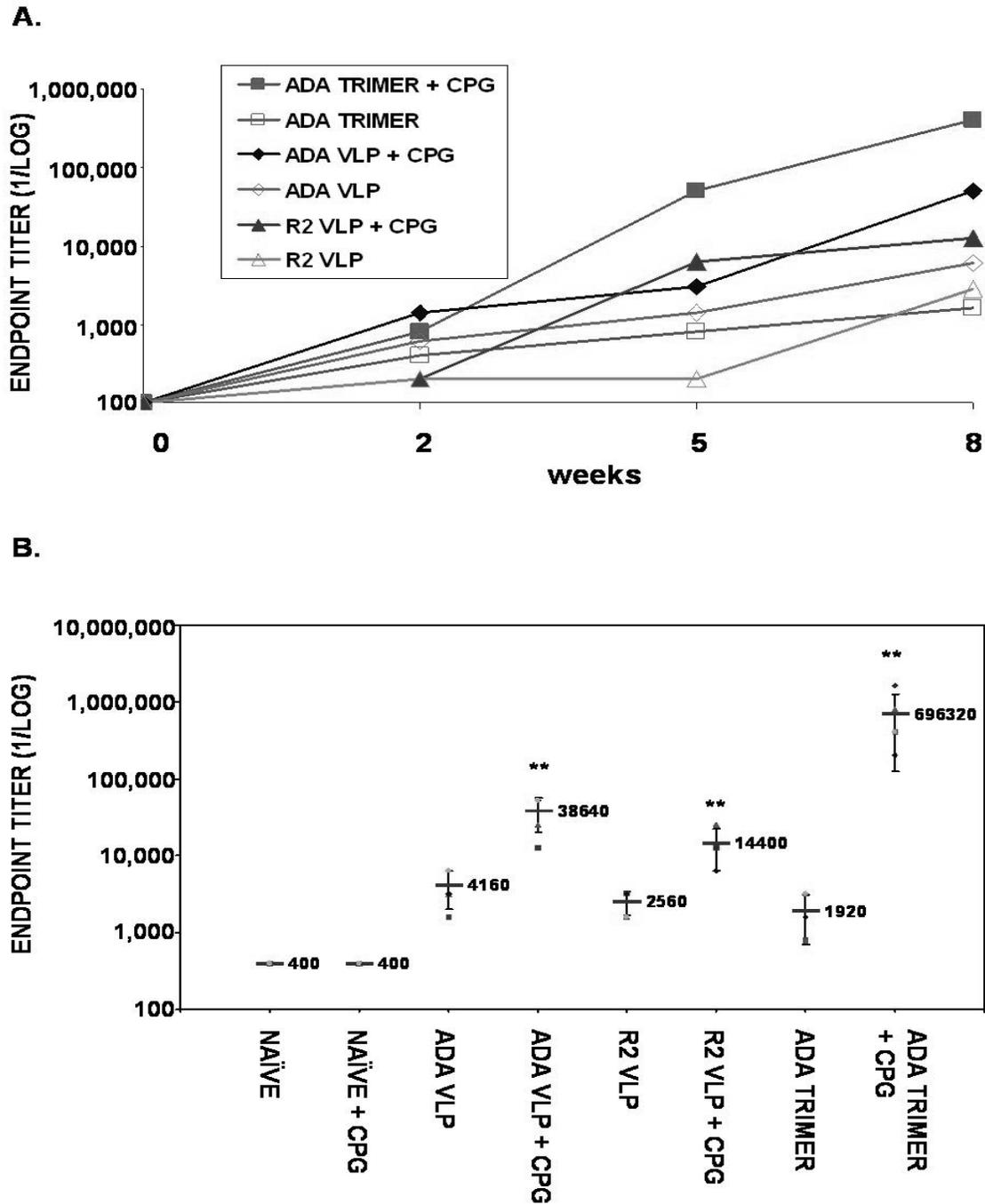


Figure 20. Anti-Env antibodies elicited by VLP-vaccinated mice.

(A) Time course of total serum IgG specific to Env for pooled serum samples. Mice were mock vaccinated or with VLPs alone +/- CpG ODNs. Serum samples were collected prior to immunization (week 0) and two weeks after each vaccination (weeks 2, 5, and 8). Anti-Env antibodies were detected by ELISA. (B) Total serum IgG for individual mice two weeks after final boost. (C) IgG isotypes were detected two weeks after final boost. Results are given as a ratio of endpoint titers of IgG_{2a} to IgG₁.

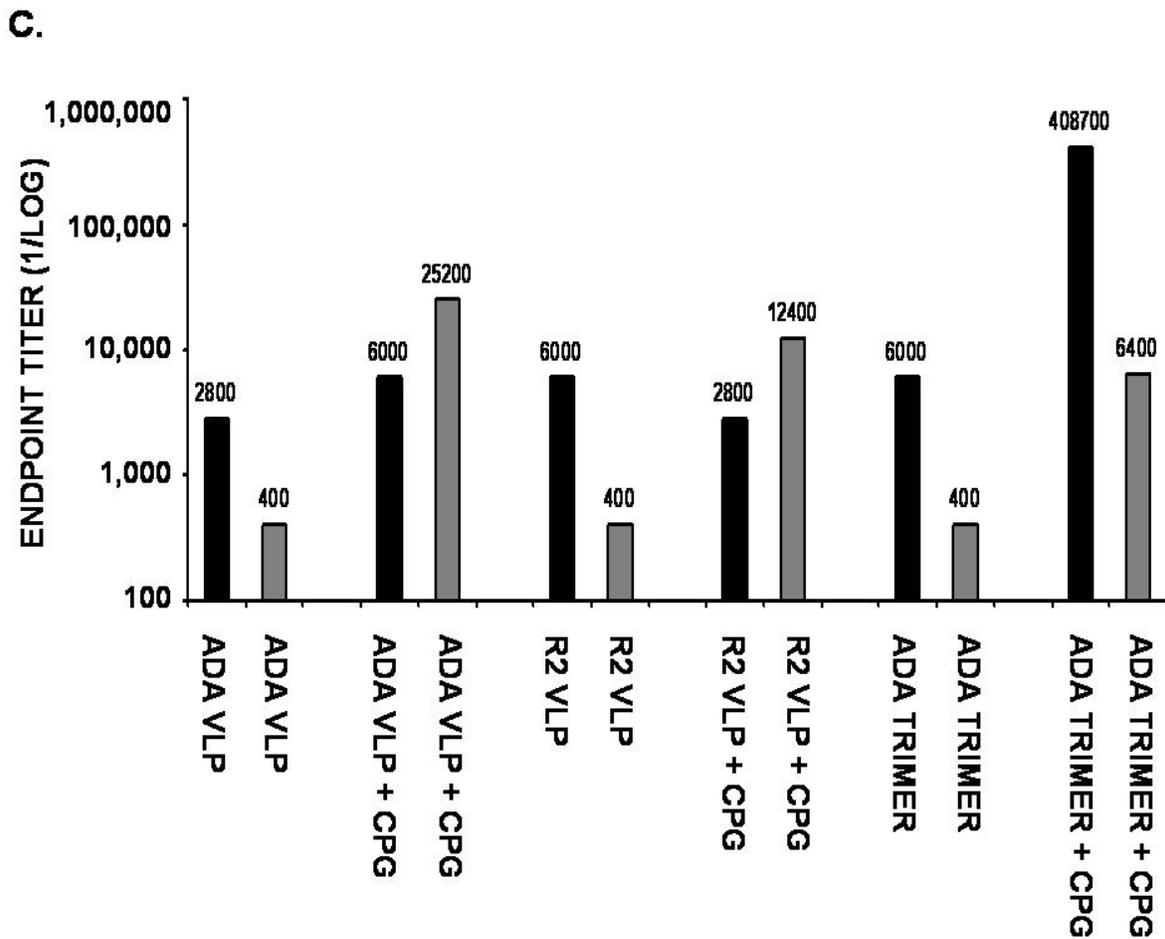


Figure 20. Anti-Env antibodies elicited by VLP-vaccinated mice.

(A) Time course of total serum IgG specific to Env for pooled serum samples. Mice were mock vaccinated or with VLPs alone +/- CpG ODNs. Serum samples were collected prior to immunization (week 0) and two weeks after each vaccination (weeks 2, 5, and 8). Anti-Env antibodies were detected by ELISA. (B) Total serum IgG for individual mice two weeks after final boost. (C) IgG isotypes were detected two weeks after final boost. Results are given as a ratio of endpoint titers of IgG_{2a} to IgG₁.

was no rise in non-specific antibodies in naïve mice or mice vaccinated with CpG ODNs only. The class of immunoglobulin elicited in the vaccinated animals differed depending if the mice were vaccinated with VLPs alone or co-inoculated with CpG ODNs (Figure 20C). Mice vaccinated with VLP plus CpG ODNs had higher IgG_{2a} titers compared to IgG₁, indicating a T helper type 1 immune response. Whereas, mice vaccinated with VLPs only had similar IgG₁ and IgG_{2a} titers, indicating a mixed T helper immune response. In contrast, the mice vaccinated with purified, soluble HIV-1_{ADA} trimers had higher titers of IgG₁ compared to IgG_{2a}. Interestingly, a Th2 response was also observed in mice vaccinated with purified, soluble HIV-1_{ADA} trimers plus CpG ODNs.

Anti-Env antibodies were also detected in mucosal immune compartments. The lung tissue of mice vaccinated with VLP had anti-Env antibodies, which were independent of CpG ODN co-inoculation (Table 8). The anti-Env titers ranged between 1:10²-1:10³ in the lungs of mice vaccinated with VLP only and 1:10³-1:10⁴ in the lungs of mice vaccinated with VLP plus CpG ODNs. Also, anti-Env antibodies were detected in distal mucosal tissues, such as the intestine and the vagina, albeit at lower titers compared to the titers detected in the lungs (Table 8). Lastly, mucosal anti-Env antibodies were detected in the lungs, vagina and intestine of mice vaccinated with purified, soluble HIV-1_{ADA} trimers alone (1:348, 1:24 and 1:24, respectively) or co-inoculated with CpG ODNs (1:16826, 1:496 and 1:56, respectively).

Sera were examined for the ability to neutralize virus infection *in vitro*⁴⁵². Mice vaccinated with VLP_{R2} (+/- CpG ODNs) or HIV-1_{ADA} Env trimers (+ CpG ODNs) were able to neutralize HIV-1_{MN} infection (Table 9). Antisera from mice vaccinated with VLP_{R2} +/- CpG ODNs (62% and 55%, respectively) or HIV-1_{ADA} Env trimer plus CpG

Table 8. Anti-Env antibodies elicited by VLP and Env trimers in mucosal tissues[†].

Vaccine group	Lungs[†]	Vagina	Intestine
VLP_{ADA}	683 ± 347	8 ± 0	8 ± 4
VLP_{ADA} + CpG	3678 ± 410	34 ± 10	56 ± 18
VLP_{R2}	274 ± 63	8 ± 0	8 ± 4
VLP_{R2} + CpG	1784 ± 256	28 ± 10	40 ± 9
Trimer_{ADA}	658 ± 356	14 ± 4	27 ± 8
Trimer_{ADA} + CpG	54877 ± 23229	1170 ± 376	82 ± 42

[†]Results from mucosal samples from naïve mice were normalized to zero and the results from the immunized mice were given as the fold increase over naïve samples.

Table 9. Neutralization of HIV-1_{MN} by anti-Env antibodies elicited by vaccinated mice.

Vaccine group	Percentage of virus neutralization [†]			
	1:20*	1:40	1:80	1:160
VLP _{ADA}	19	3	45	19
VLP _{ADA} + CpG	22	30	26	26
VLP _{R2}	37	23	62	41
VLP _{R2} + CpG	25	14	55	30
Env _{ADA} trimer	12	0	0	0
Env _{ADA} trimer + CpG	46	43	49	47

[†]Results from individual sera samples from naïve mice were normalized to zero neutralization. *The percentage of virus neutralization was determined multiple dilutions (1:20, 1:40, 1:80 and 1:160). Sera from vaccine groups exhibiting >50% virus neutralization are shaded.

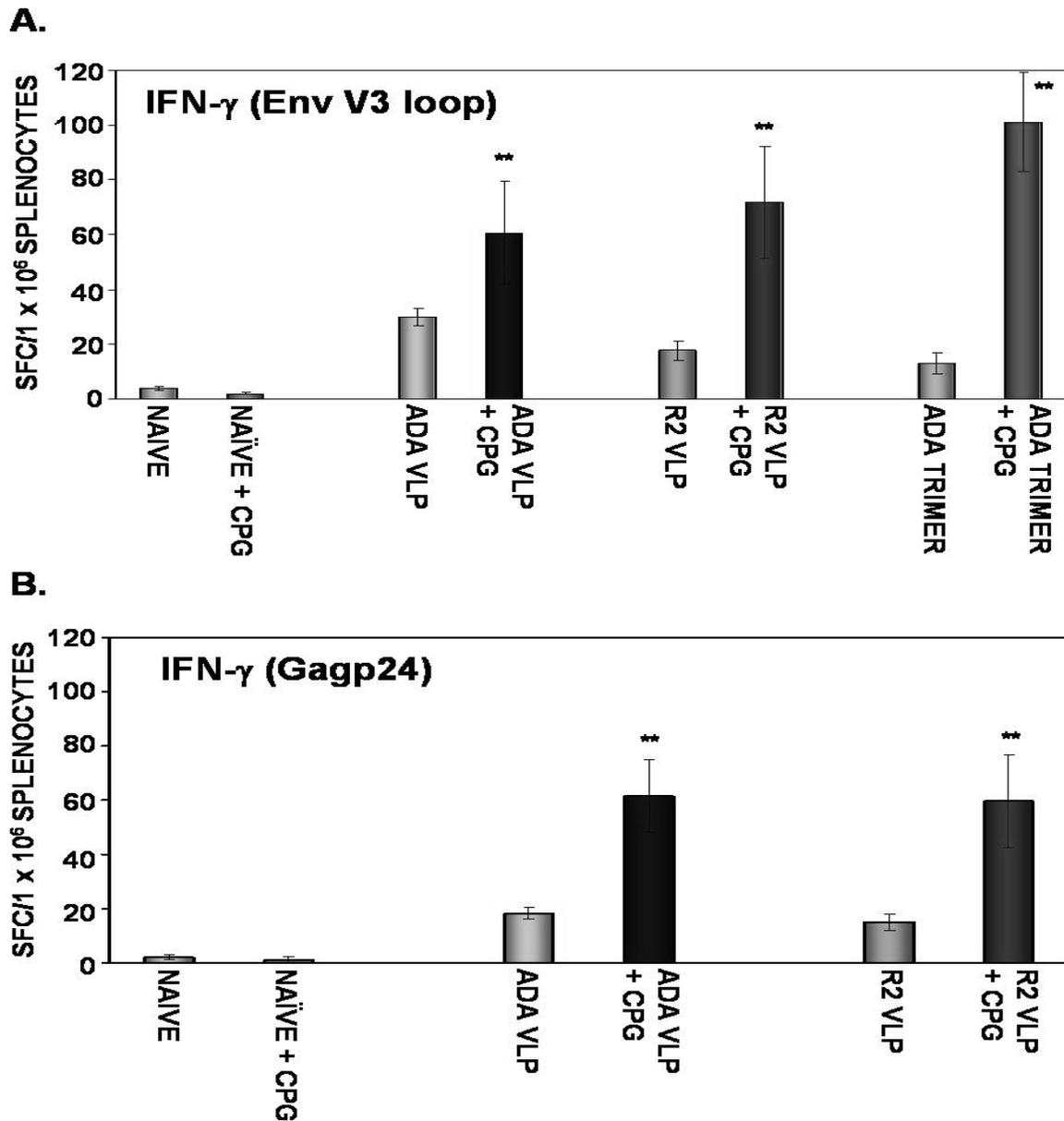


Figure 21. HIV-1 Gag and Env peptide-specific T-cell response after immunization of mice with VLPs.

BALB/c mice were immunized intranasally with VLPs +/- CpG ODNs or mock vaccinated. Two weeks after the final boost, splenocytes were prepared and stimulated with (A) HIV-1 Env_{gp120(V3 loop)} or (B) Gag_{p24} peptides. HIV-specific IFN- γ production was detected by an ELISPOT assay. As a negative control, cells from naïve mice produced none or less than 7 spots per 1 million splenocytes. The number of background spots was subtracted from the number of spots in the sample wells (<5 spots per 1 million splenocytes). Error bars, SEs for 5 mice in a group. The statistical significance of the difference between groups was calculated by Student's *t* test.

ODNs (49%) neutralized >50% of HIV-1_{MN} (1:80 dilution). In contrast, the mice vaccinated with VLP_{ADA} (+/- CpG ODNs) or HIV-1_{ADA} Env trimers alone did not elicit neutralizing antibodies at the examined dilution of sera.

Cellular immune response elicited by VLPs

Mice vaccinated with VLP plus CpG ODNs elicited higher numbers of INF- γ secreting cells compared to mice vaccinated with VLP only (Figure 21). Splenocytes collected from mice vaccinated with VLP plus CpG ODNs had a higher number of cytokine secreting cells following stimulation with a set of MHC class I restricted (V3 loop) peptides compared to mice vaccinated with VLP alone (Figure 21A). Mice vaccinated with purified, soluble HIV-1_{ADA} trimers alone or co-vaccinated with CpG also elicited splenocytes secreting IFN- γ following stimulation with the same V3 loop peptides (13 and 101 spots/10⁶ splenocytes, respectively). In comparison, splenocytes stimulated with the peptides specific for Gag_{p24} collected from mice vaccinated with VLP plus CpG ODNs had 3 times the number of INF- γ secreting cells as mice vaccinated with VLP alone (Figure 21B). Naïve, age-matched mice, as well as unstimulated splenocytes from mice vaccinated with VLP +/- CpG, had few INF- γ secreting splenocytes (0-7 spots) following *in vitro* re-stimulation with either set of peptides.

V.D. Discussion

In this study, mice were inoculated intranasally with purified human

immunodeficiency virus-like particles. VLPs were purified via transient transfection of COS cells followed by ultracentrifugation through 20% glycerol (Figure 19). Each VLP elicited specific immunity to HIV-1 antigens in both the systemic and mucosal immune compartments. Anti-Env antibodies were detected in the sera (Figures 20A and B), as well as in the washes from harvested lungs, intestines, and vagina from immunized mice (Table 8). In addition, Env and Gag specific IFN- γ -secreting splenocytes were elicited in the mice vaccinated with VLPs (Figure 21). Co-inoculation of CpG ODNs with VLPs significantly enhanced both sets of the immune response. Additional mice were intranasally vaccinated with a high dose of purified, soluble HIV-1_{ADA} trimers (+/-) as a positive control. These mice elicited strong humoral and cellular responses when co-immunized with CpG ODNs, but minimal responses were detected in mice vaccinated with purified, soluble HIV-1_{ADA} trimers alone. In contrast to the VLP-vaccinated mice, the main IgG isotype detected in the mice immunized with purified, soluble HIV-1_{ADA} trimers was IgG₁ regardless if CpG ODNs were co-inoculated.

Virus-like particles, or pseudovirions, can be defined as self-assembling, non-replicating, non-pathogenic, genomeless particles that are similar in size and conformation to intact virions. These immunogens can be administered as purified particles or as DNA plasmids expressing the viral proteins to form VLPs *in vivo* (for reviews, see references ^{149, 719}). Several experimental vaccines for HIV/AIDS use a multi-gene strategy to elicit immunity, however, these vaccines do not produce VLPs with Env embedded in the particle membrane ^{326-328, 383, 384, 468}. Many of the lentiviral-like particles currently under investigation utilize the recombinant vaccinia virus (VV) or baculovirus (BV) systems for particle purification ^{81, 82, 210, 245, 487, 666, 667, 714}. Cellular and

humoral immune responses have been elicited using lentiviral VLPs produced from cells infected with rVV or rBV expressing SIV or HIV *gag* and *env* genes or cell lines stably expressing both genes^{307, 308, 715}. However, these purification systems have limitations, which may limit their use. Some of the advantageous characteristics of the HIV-1 multi-gene VLP vaccine used in this study are the VLP: 1) induces both of humoral/cellular and systemic/mucosal immune responses, 2) is expressed from a virally regulated gene insert, 3) is a single plasmid composed of multiple gene products with native conformation(s) of surface envelope⁵⁶³, 4) can transduce antigen presenting cells, 5) can be processed by these APCs for presentation of viral peptides on both class I and II MHC molecules, 6) glycosylation by primate rather than insect cells and 7) has increased safety due to the lack of replication competence.

Each VLP was expressed from a single gene insert that contained *gag*, *pol*, *env*, *vpu*, *tat* and *rev* gene sequences⁷²⁰. In addition, each VLP is non-infectious due to the deletion of the sequences encoding IN, Nef, Vpr, and Vif and the LTRs (Figure 7) and⁷²⁰). Several mutations were introduced into the VLP genomic DNA to abrogate reverse transcriptase and RNase H activities and to restrict RNA packaging to increase the safety of these immunogens for potential use in humans. Each VLP expressing DNA was transfected into both primate and mouse cell lines (Figure 19a), however, only primate cells secreted particles from cells. Several reports have indicated that HIV-1 is unable to replicate in rodent cells, due to blocks in entry and fusion, integration, gene expression, nuclear transport, viral budding, and maturation (For review, see reference⁶⁴⁸). Not surprisingly, murine cells transfected with DNA plasmid expressing the VLP gene insert had little or no gene products in the lysate fraction and no particles were detected in the

supernatants of transfected cells (Figure 19b). We introduced two tandem copies of the MPMV CTE^{611, 613, 614} to the 3' end of the VLP insert (VLP_{2xCTE}). Nuclear export and subsequent protein expression was enhanced in murine cells transfected with VLP_{2xCTE}. However, no particles were detected in the supernatant indicating that the VLPs were still blocked from budding. Therefore, a DNA vaccination strategy using these VLP expressing plasmids was not an option for *in vivo* particle secretion in mice.

VLPs were purified from supernatants of monkey cells transiently transfected with VLP expressing DNA and used to vaccinate mice via intranasal inoculation for the elicitation of immune responses. Anti-Env antibody titers rose after each vaccination. These anti-Env antibodies were elicited by envelope on the surface of the VLP, since free Env was filtered out during the purification process. Free proteins, with a density of less than 1.081 g/ml, will not penetrate 20% glycerol purification during ultracentrifugation⁷²⁰, therefore, only Env, retained on the surface of the VLP, stimulated anti-Env immunity. Even though envelope was detected on the surface of the purified VLP at very low levels (10 ng/ml) by ELISA, anti-Env antibodies were elicited by envelope on the surface of the VLP in vaccinated mice. The elicited antibodies were specific for envelope, not against cellular monkey proteins embedded in the VLP membrane, since sera from mice vaccinated with Gag only particles (no envelope) elicited low levels of antibodies that cross-reacted with Env.

Recent studies have indicated that uncleaved Gag gene products retain Env on the surface of a particle more efficiently than fully processed Gag molecules^{250, 705}, similar to these VLPs. An uncleaved Gag particle core may enhance Env retention by providing conformational constraints on the ectodomain of the HIV-1 envelope glycoprotein,

leading to enhanced envelope stability⁷⁰⁴. In addition, these findings are supported by the detection of envelope on the surface of VLPs by electron microscopy using anti-Env immunogold staining of VLPs derived using similar expression systems^{173, 174, 603}. In order to develop VLPs as a vaccine platform for Env presentation, the use of an immature particle cores to avoid Env loss may be advantageous. In addition, the combination of enhanced stability with a highly immunogenic envelope that elicits broadly cross-reactive, multi-clade immunity to native Env epitopes would advance AIDS vaccine development.

The *env* gene segments were cloned from two R5 isolates (ADA and R2) into the VLP_{BH10} genome for their unique neutralization properties. CXCR4 utilizing envelopes, such as the Env_{BH10}, are more easily shed from the particle surface and are more sensitive to neutralizing antibody compared to primary R5 isolates^{116, 437} and therefore, they have not proven effective immunogens for AIDS vaccine development. The Env_{ADA} is a particularly difficult envelope to neutralize and therefore, vaccine strategies that elicit neutralizing antibodies using VLP expressing this envelope may elicit a broader immune response to a diverse set of HIV-1 isolates. Only mice vaccinated with VLP_{R2} had neutralization titers (50% inhibition at a 1:80 dilution). Env_{R2} is a CD4-independent envelope that efficiently elicits neutralizing antibodies^{151, 721}. However, the analysis of the neutralizing capacity of the elicited antibodies was compromised due to the well-documented, non-specific neutralization components in mouse antisera⁴⁵², which affected our ability to measure the neutralization at lower dilutions. Future studies using these VLPs in rabbits or non-human primates should yield more definitive results.

In addition to the induction of antibodies in the peripheral immune system, anti-Env antibodies were detected in mucosal tissues (Table 8). Not surprisingly, the lungs had the highest antibody titers compared to mucosal sites further from the inoculation site. The NALT contains all the immunocompetent cells required to generate an effective immune response. Immune responses in the NALT are connected to the common mucosal immune system through inductive distal sites such as the lamina propria of the intestinal tract or urogenital tissues for the generation of both antibody and T cell responses¹²⁸. However a high variability was noted in the total sIgA, which was not observed in the mucosal anti-Env IgG titers.

Compared to particulate antigens, such as VLPs, intranasal vaccination of soluble proteins, in the absence of an adjuvant, induces low or undetectable immune responses in rodents and primates^{274, 430, 460, 637}. In this study, intranasal immunization of VLPs induced both systemic and mucosal immunity (Figures 20 and Table 8) Soluble antigens can penetrate the nasal epithelium and directly interact with dendritic cells, macrophages and lymphocytes and then they are transferred to posterior lymph nodes⁶³². In contrast, in this study, VLPs were most likely phagocytosed by microfold epithelial cells (M cells) in the nasal lumen and then directly deposited to the NALT (nasal associated lymphoid tissue) via M cell transcytosis⁶⁹⁷, which preferentially drains into cervical lymph nodes. This process induces strong local (NALT) and distant immune responses in both peripheral and mucosal immune compartments⁶⁰¹. Soluble antigens bypass the NALT and are directly fed into superficial lymph nodes by antigen presenting cells in the nasal lumen resulting in a lower local immune response⁶⁰¹. Therefore, VLP immunogens directly interact with the mucosal immune system to elicit high titer immunity.

Both humoral and cellular immune responses were enhanced in mice vaccinated with VLPs plus CpG ODNs. Splenocytes collected from mice vaccinated with purified VLPs had robust anti-Env and anti-Gag responses (Figure 21), which were increased 2-3 fold in mice co-administered with CpG ODNs. The numbers of splenocytes reacting to Env or Gag peptides were significant, especially since only 2 overlapping peptides were used for each antigen. Overall, mice vaccinated with VLPs elicited cellular responses against Gag and Env, which were enhanced by the co-immunization of CpG ODNs.

For most protein or particle immunizations, adjuvants are necessary in order to elicit robust immune responses³⁵⁰. Several compounds have been used in rodent and non-human primate studies to enhance the mucosal immunity, such as cholera toxin B (CT) and *Escherichia coli* heat-labile enterotoxin (LT)^{21, 158, 159, 197, 637}, QS21^{320, 565}, monophosphoryl lipid A (MPL)^{241, 242, 646}, and CpG ODNs^{334, 335, 337, 338, 350, 431}, as well as cytokines, such as GM-CSF and RANTES^{65, 307, 388, 490, 715}. In this study, CpG ODNs were used to boost the immunogenicity elicited by intranasal administration of VLPs because they: 1) are potent inducers of both humoral and cellular mucosal immune responses²⁵⁵, 2) have reduced toxicity compared to CT or LT^{271, 676}, and 3) induce both mucosal and systemic immune responses³⁵⁰. These adjuvants are derived from bacterial DNA containing unmethylated cytosine-phosphate-guanosine motifs, which are usually inoculated in the form of synthetic oligonucleotides (CpG ODNs). DNA sequences containing one or more CpG motifs are endocytosed and activate the toll-like receptor, type 9 (TLR9), which are found on human B cells and plasmacytoid dendritic cells^{244, 264, 625}. VLP plus CpG ODN vaccinated mice had a T helper type 1 response compared to mice vaccinated with VLPs only, as indicated by a higher IgG_{2a}:IgG₁ ratio (Figure 20c).

Most likely, mice vaccinated with VLPs plus CpG ODNs had heightened B cell activation^{249, 339, 353} resulting in increased antigen presentation and antibody production, as well as increased T cell activation, antigen presentation by B cells and plasmacytoid dendritic cells²⁴⁰ and enhanced lytic activity by INF- γ secreting NK cells^{230, 378, 550}. Therefore, even though VLPs alone were immunostimulatory, the addition of the CpG ODN adjuvant enhanced both humoral and cellular responses, which should be considered for future primate experiments.

In summary, VLPs, derived from a virally-regulated, multi-gene plasmid DNA, elicit immune responses to HIV-1 antigens in both the peripheral and mucosal immune compartments. These VLP immunogens can be expressed *in vivo* from a single plasmid or from viral/bacterial vectors or administered as purified particles. The elicitation of immune responses at the mucosa is a goal of AIDS vaccine development, because the vast majority of HIV-1 transmission is through sexual contact. Exposure of the vagina or rectal tissue to HIV-1 infected seminal fluid effectively transmits the virus, and the induction of urogenital mucosal immunity against HIV-1 could enhance protection. Intranasal inoculation of VLPs to induce urogenital tract mucosal immune responses is an attractive and practical approach for administration of an AIDS vaccine.

V.E. Acknowledgments

This research was supported by National Institute of Health Grant AI51213 to T.M.R. The authors thank Narasimhachar Srinivasakumar for supplying the MPMV 2xCTE and Gerald Quinnan for providing the HIV-1_{R2} envelope gene. The authors

would like to thank Joseph Bower, Franklin Toapanta (technical assistance and critical review of the manuscript), Velpandi Ayyavoo, Kelly Cole, Sharon Hillier, and Ronald Montelaro (helpful discussions). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (also called JC57BL-13) (#8129), HIV-1_{MN} (#317), HIV-1_{ADA} (#416), HIV Ig (#3957, from NABI and NHLBI).

VI. Chapter 6: Specific Aim III

HIV-1 Gag Particles Elicit Broader Immunity Compared to Soluble, Non-Particulate or Intracellularly-Confined Gag Proteins

VI.A. Introduction

Gag gene products, assisted by cellular machinery and host proteins, are the driving force behind viral assembly into particles. HIV-1 *gag* encodes for the major structural viral proteins: capsid (CA, p24), matrix (MA, p17), and nucleocapsid (NC, p7) (Figure 22). Similar to all lentiviruses, HIV-1 *gag* and *pol* are expressed from full-length, unspliced mRNA, which also serves as genome for nascent virions. Both unspliced and singly spliced viral mRNAs are transported from the nucleus to the cytoplasm by the virally encoded protein, Rev^{409, 411, 412, 723}. Rev recognizes an element in the viral RNA, RRE, which facilitates the efficient transport of incompletely spliced mRNAs^{184, 608}. However, optimizing *gag* or *pol* genes to express the codons most often found in primate cells (codon optimization) circumvents the Rev-dependent nuclear export of these mRNAs¹³⁸.

Gag is expressed as a 55 kDa precursor (Gag_{p55}) and is cleaved by the viral protease (PR) to produce distinct Gag gene products (MA, CA, p2, NC, p1, and p6^{Gag}) (Figure 22). Pol is synthesized as part of the large ~160 kDa polypeptide (Gag-Pol_{p160}) by a ribosomal frame slip (-1 in the *gag* reading frame) at the *gag-pol* junction, which occurs 5-10% of the time during protein synthesis²⁹³. The Gag-Pol_{p160} molecule is cleaved to produce protease (PR), reverse transcriptase (RT), and integrase (IN), as well as the Gag gene products. Translation of unspliced mRNAs results in the formation of both Gag_{p55} and Gag-Pol_{p160} at a 20 to 1 ratio, which is critical for particle formation and infectivity^{293, 313, 502, 587}. Gag_{p55} can self-assemble and form VLPs, however, Gag-Pol_{p160} cannot form a virus-like particle alone, but can be incorporated into particles through interactions with CA domains in Gag_{p55}^{97, 154, 156, 314, 315, 536}.

Gag_{p55}

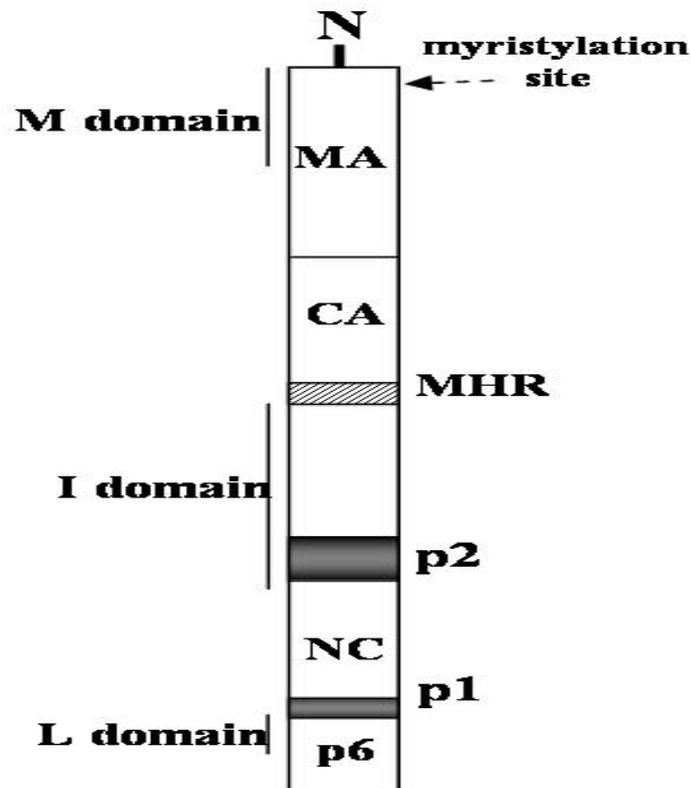


Figure 22. Schematic representation of the major features of Gag_{p55}.

The schematic represents the Gag_{p55} gene sequence. The major Gag gene products are linearly represented; matrix (MA), capsid (CA), nucleocapsid (NC) and p6 (N-terminus, top and C-terminus, bottom). The two spacer domains, p1 and p2, are located in between NC/p6 and CA/NC, respectively. The major homology region (MHR) and basic domain are indicated along with the membrane-binding (M), Gag-Gag interaction (I) and late (L) domains. The arrow denotes the myristylation site, which was mutated (glycine to alanine, gga to gca) by site directed mutagenesis PCR.

Assembly pathways differ between cell types with viral particles budding from the plasma membrane in T lymphocytes, while viral assembly occurs in intracellular vesicles in macrophages^{75, 194, 229, 294, 497}. Regardless of the location of assembly, Gag_{p55} produces multimers by binding to one another at the major homology region (MHR) found in CA, and post-translational myristylation of the N-terminus of Gag_{p55} targets the polyprotein aggregates to the membrane. Gag attaches to the membrane via the myristic acid moiety and is secured by a stretch of basic amino acids in MA^{491, 492, 496, 722, 728, 729}. Following assembly, the budding of immature Gag particles resemble electron-dense, donut-shaped cores, containing uncleaved Gag_{p55} polyproteins^{245, 666}.

Although the correlates of protections are unclear, the main structural proteins encoded by *gag* are major targets of the immune response^{139, 662, 663}. Most current HIV/AIDS vaccine clinical trials incorporate a Gag component into the vaccine design (for reviews please see^{610, 719}). Gag gene products have been expressed *in vivo* from viral vectors, DNA plasmid, or live-attenuated virus^{127, 225, 287, 379-381, 397, 512, 544, 594}. In addition, whole-inactivated virions, virus-like particles, or soluble Gag gene products have been administered by parenteral and mucosal inoculation^{141, 509, 662, 719}. Gag proteins, as components of these vaccine strategies, elicit strong humoral and cellular immune responses.

Particle-based vaccine approaches for HIV-1 are particularly attractive strategy because particulate antigens have a longer half life than soluble antigens. In addition, viral particles enter different antigen presentation pathways and trigger “danger signals” to stimulate a more robust immune response. Particles administrated mucosally transverse microfold epithelial cells (M cells) and enter the associated lymphoid tissue⁶⁹⁷. The associated lymphoid tissues preferentially drain into the regional lymph nodes and this process induces strong local and

systemic immune responses⁶⁰¹. In contrast, soluble antigens remain at the inoculation site or bypass the associated lymphoid tissues and directly enter the superficial lymph nodes⁶³². Particulate antigens that enter and interact with this highly concentrated area of immune cells may lead to an early and more robust immune response compared to soluble antigens that bypass this region. However, the modality, dose or route of inoculation, immunogens co-inoculated with Gag proteins, use of adjuvants, or prior exposure to the pathogen or vaccine appears to influence the effectiveness of immunization^{4, 141, 260, 454, 499, 552, 555, 559, 635, 659, 663}. In addition, the structural form of the Gag gene products may influence the effectiveness of the elicited anti-Gag immunity in order to protect a host from disease.

Therefore, the goal of this study was to compare the elicitation of immune responses by the same Gag immunogens presented to the immune system in different structural forms. Mice were inoculated with vaccines expressing HIV-1 Gag gene products *in vivo* as DNA expressing Gag particles or proteins retained intracellularly. In addition, the immunity elicited by purified Gag_{p55} particles (virus-like particles) in mice was compared to denatured, soluble Gag_{p55} proteins.

VI.B. Material and Methods

DNA Plasmids

The vaccine plasmid, pTR600, has been previously described^{62, 224, 446, 634, 720}. Briefly, the vector was constructed to contain the CMV-IE for initiating transcription of eukaryotic inserts and the BGH poly A for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (*kan^r*) for

selection in antibiotic media.

The previously described plasmid, pGag, expresses Gag_{p55} VLPs composed of unprocessed Gag_{p55} polyprotein (isolate HIV-1_{NL4-3}) from codon-optimized DNA sequences^{283, 720}. In this study, virus-like particles produced *in vitro* were purified from COS cells transfected with the pGag. The plasmid, pGag_{ΔMyr}, was constructed by mutating the myristylation site (second amino acid in MA_{p17}) by site-directed mutagenesis PCR (Quick Change XL Kit, Stratagene, Cedar Creek, TX). This mutation resulted in a change of the codon from a glycine to an alanine (gga to gca), which was verified by DNA sequencing. The plasmids were amplified in *Escherichia coli* strain-DH5α, purified using anion-exchange resin columns (Qiagen, Valencia, CA) and stored at -20°C in dH₂O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260nm and 280nm.

Expression of DNA plasmids

Monkey (COS) or murine (NIH 3T3) fibroblastic cell lines were transfected (5×10^5 cells/transfection) with DNA (2 μg) using lipofectamine (12%) according to the manufacturer's guidelines (Life Technologies, Grand Island, NY, USA). Supernatants (2 ml) and cell lysates (300 μl dH₂O) were collected and stored at -20°C. For western blot analysis, samples were diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA, USA), boiled (5 min), and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and incubated with polyclonal human HIV-infected patient antisera (HIV Ig) (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk). After extensive washing, bound human antibodies were detected with horseradish

peroxidase-conjugated goat anti-human IgG antiserum (1:10,000 in PBS containing 0.05% Tween 20 and 5% non-fat dry milk) and enhanced by chemiluminescence (Amersham, Buckinghamshire, UK).

Purification of Gag_{p55} virus-like particles

Supernatants from COS cells, transiently transfected with pGag or pGag_{ΔMyr}, were pelleted (2 h at 4°C) via ultracentrifugation (100,000 x g through 20% glycerol, v/v). The particles were subsequently resuspended and used for 1) *in vivo* inoculation or 2) sucrose gradient ultracentrifugation. Gag_{p55} particles used for immunization were resuspended in sterile PBS. Total protein concentration was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). In addition, the level of non-HIV protein contamination (from the media/cells) in each inoculum was visualized by a silver-stained SDS-PAGE using the ProteoSilver Silver Stain Kit (Sigma, St. Louis, MO, USA). The specificity of each inoculum was analyzed in a separate immunoblot that was probed with HIV Ig as described earlier.

Pellets were analyzed for particle content by ultracentrifugation through 20-60% sucrose gradients (11 steps, 4% increments) (17 h at 100,000 x g at 4°C). Eleven fractions (20-60% sucrose, 1 ml, weight per volume in PBS) were collected (top to bottom) from the gradient, and the proteins were precipitated with equal volumes of 20% TCA and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by HIV Ig via western hybridization and visualized by enhanced chemiluminescence. In addition, the purity of each VLP preparation in each inoculum was visualized by a silver-stained polyacrylamide SDS gel .

Mouse immunizations

Female BALB/c mice (5–7 weeks old) were immunized (days 1, 21, and 42) with one of two DNA immunogens, pGag and pGag_{ΔMyr} (5 μg), or soluble Gag_{p55} protein (10 μg) purified from yeast lysates (50mM sodium phosphate, 0.4 M NaCl, 6M Urea, ph 7.9, AIDS Reagent and Reference Program), or primate cell-derived Gag_{p55} virus-like particles (40 μg total protein) via intramuscular immunization. The amount of Gag_{p55} virus-like particles used for immunization was normalized to the amount of VLPs expressed from pGag and the same amount of soluble Gag_{p55} as determined by ELISA. Soluble Gag_{p55} protein and Gag_{p55} VLPs were co-inoculated with phosphorothioate CpG oligodeoxynucleotides (CpG ODNs, 10 μg each). Each CpG ODN (ODN-1: 5'-TCCATGACGTTCTGACGTT-3', ODN-2: 5'-TGACTGTGAACGTTTCGAGATGA-3')^{203, 274, 307, 428, 451} was synthesized and purified by high-pressure liquid chromatography (Sigma-Genosys, The Woodlands, TX, USA). The CpG ODNs were resuspended in sterile water (2 μg/μl) and stored at -80°C. All animal groups were intramuscularly administered (1X) with the respective vaccine in sterile PBS into the left quadriceps muscle (50 μl total volume).

Mock DNA vaccinated (pTR600 vector) or CpG ODN inoculated mice served as negative controls. For mock VLP, supernatants from primate cells transiently transfected with the vector, pTR600, were ultracentrifuged through 20% glycerol. The pellet was resuspended in sterile PBS plus CpG ODNs and inoculated into the naïve mice (equivalent volume compared to mice given a VLP inoculum). Mice were anesthetized with xylazine (20 mg/ml) and ketamine (100 mg/ml) administered subcutaneously in the abdomen prior to immunization and blood collection. Mice were housed in compliance with U.S.D.A. regulations and were monitored daily for weight loss, behavior, and adverse reaction.

Antibody responses to Gag proteins

Blood samples were collected by retro-orbital plexus puncture (days 1, 35, and 56 post-immunization) on anesthetized mice. Sera samples were separated from red blood cells by centrifugation (5,000 rpm, 10 min) and stored at -80°C. Sera were tested for antibodies to Gag_{p55} by ELISA. Each well of a 96-well plate was coated with 100 ng of purified Gag_{p55} (NIH ARRRP) (4°C for 16 hr). Plates were blocked (25°C for 2 hr) with PBS containing Tween 20 (0.05%) and nonfat dry milk (5%), and serial dilutions of each sample were incubated (25°C for 2 hr). Following thorough washing in PBS-Tween 20 (0.05%), samples were incubated (25°C for 1 hr) with biotinylated goat anti-mouse IgG (1:5,000) diluted in PBS-Tween 20 (0.05%) and nonfat dry milk (5%). The unbound antibody was removed, and the wells were washed. Streptavidin-HRP (1:7,000) was diluted in PBS-Tween 20 (0.05%) and incubated (25°C for 1 hr). Samples were incubated with TMB substrate (1 hr), and the colorimetric change was measured as the optical density (O.D., 405nm) by a spectrophotometer (Dynex Technologies, Chantilly, VA, USA). The value of naïve sera was subtracted from the test samples.

ELISPOT assay

Isolated spleens (week 8) were minced using a rubber stopper and mesh gauze using sterile PBS to generate a single cell suspension. Splenocytes were depleted of erythrocytes by treatment with ammonium chloride (0.1 M, pH 7.4). Following thorough washing with PBS, cells were resuspended in RPMI medium with 10% fetal bovine serum (cRPMI). Cell viability was determined by trypan blue exclusion staining. The number of anti-Gag specific murine INF- γ (mINF- γ) secreting splenocytes was determined using an ELISPOT assay kit (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-mINF- γ plates were incubated (25°C for 2 hr) with cRPMI (200 μ l) and then were incubated with splenocytes (10^6 /well) isolated from

vaccinated mice. Splenocytes were stimulated (48 h) with 2 overlapping peptides representing an immunodominant CTL epitope for HIV-1 Gag_{p24} (HIV-1 consensus B Gag: GHQAAMQMLKETINE and AMQMLKETINEEAAE) or purified Gag_{p55} purified protein (1 µg/well of each peptide or 2 µg protein). Additional wells of splenocytes were stimulated with PMA (50 ng)/ionomycin (500 ng) or were mock stimulated. In addition, IL-2 was added to all wells (10 units/ml). Plates were washed with PBS-Tween (3X) and incubated (25°C for 2 h) with biotinylated anti-mIFN-γ and incubated (4°C for 16 h). The plates were washed and incubated (25°C for 2 h) with streptavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25°C for 1 h) with stable BCIP/NBT chromagen. The plates were rinsed with sterile water and air dried (25°C for 2 h). Spots were counted by an ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA). Results are given as the number of spots per 10⁶ splenocytes after subtracting the background values (naïve mice stimulated with Gag peptides or proteins and unstimulated splenocytes from test samples).

Cytotoxicity assay

At week 8, splenocytes were harvested and sensitized with the same immunodominant CTL peptides and Gag_{p55} protein (2 µg/ml) as described for the ELISPOT assay. This mixture was incubated with the splenocytes for 7 days (37°C, 5% CO₂) with 10 units/ml murine IL-2 in cRPMI. At day 4, additional media was added to the samples (Final concentrations: 1µg/ml of peptides and protein, 5 units/ml of IL-2). One week later, effector cells were assayed for cytolytic activity in a non-radioactive CTL assay using two BC10ME cell lines stably expressing HIV-1 Gag or Pol. These stably transfected cell lines have been shown to be more sensitive as

target cells compared to peptide-pulsed BC10ME cells in a previous CTL assays²⁸³. Mouse splenocytes (10,000-800,000 effector cells) and BC10ME_{Gag} or Pol cells (10,000 target cells) were incubated for 5 h (ET ratio = 1:1-1:80). Cytotoxicity was measured quantitatively by the release of LDH from lysed cells using a CytoTox 96 Non Radioactive Cytotoxicity Assay kit according to the manufacturer's protocol (Promega, Madison, WI). This non-radioactive cytotoxicity assay demonstrates similar results compared to ⁵¹Cr release assays^{132, 342}. Results are given as: % Cytotoxicity = [(Experimental - Effector Spontaneous - Target Spontaneous) ÷ (Target Maximum - Target Spontaneous)] X 100.

VI.C. Results

Characterization of Gag_{p55} immunogens

Four Gag immunogens were designed to examine the elicitation of immune responses by the same antigen presented to the immune system in different forms. Two DNA vaccines containing the same gene sequences were constructed to express Gag_{p55} proteins *in vivo*. The previously described plasmid, pGag⁷²⁰, expressed Gag_{p55} VLPs composed of Gag_{p55} polyprotein from HIV-1_{NL4-3} gag sequences (Figure 22), which have been optimized for expression in mammalian cells. The plasmid, pGag_{ΔMyr}, was constructed from pGag. The myristylation site located at the second amino acid (glycine) in the matrix gene (Gag_{p17}) was mutated to an alanine, which disrupts the stable association of viral proteins with the membrane components during the assembly process and therefore prevents the formation of particles^{354, 492, 496, 722, 728, 729}. Two additional Gag immunogens were compared for the elicitation of anti-Gag_{p55} immunity. These

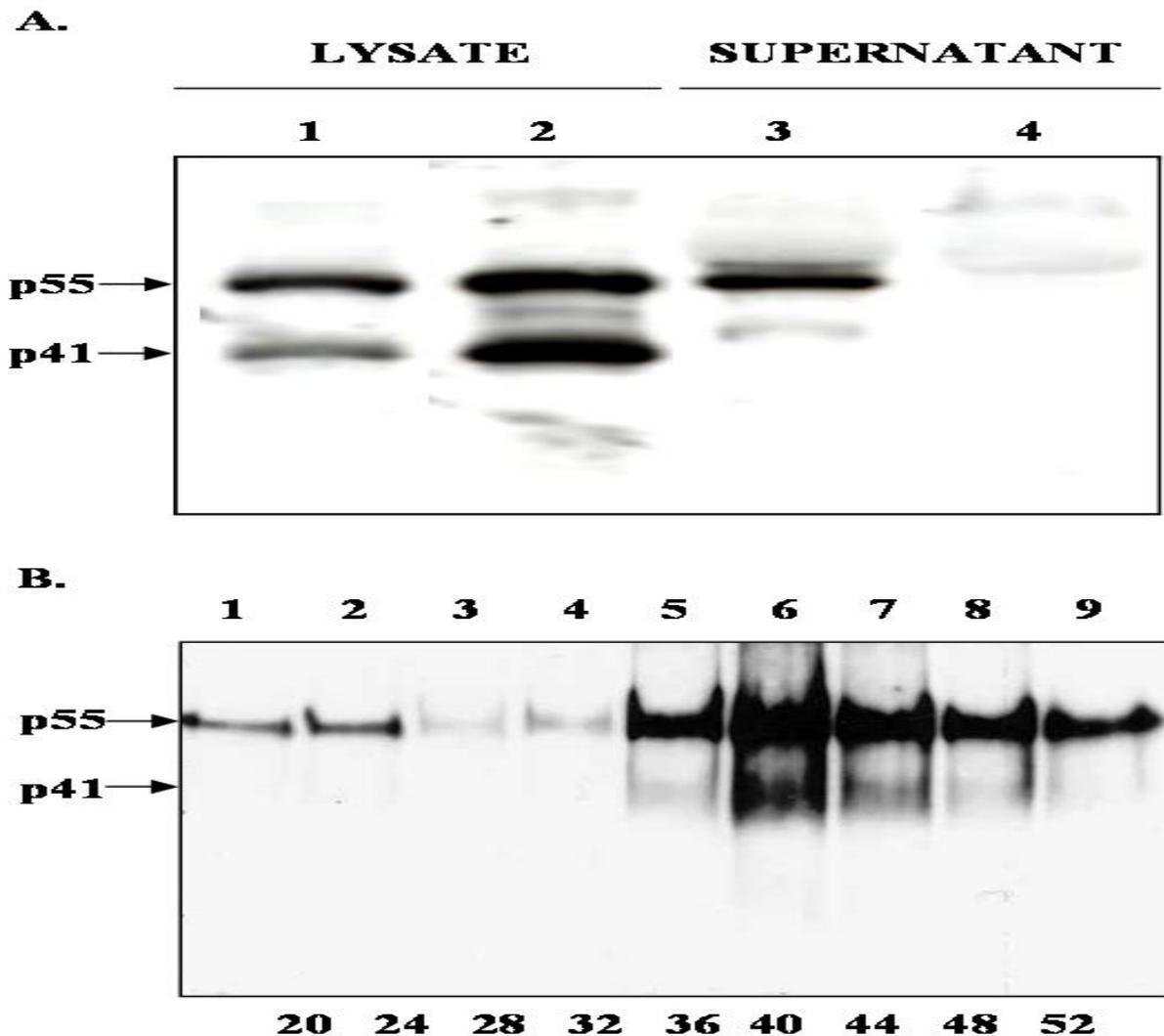


Figure 23. Expression of Gag proteins in primate and rodent cells.

(A) Protein expression of vaccine plasmids. NIH 3T3 (murine) cells were transfected with 1 μ g of each vaccine plasmid and protein expression was verified by western blot analysis. Lanes 1, 3: pGag and lanes 2, 4: pGag $_{\Delta MYR}$. Lanes 1, 2: 2% cell lysates and lanes 3, 4: 1% of supernatants of NIH 3T3 cells. (B) Sucrose density gradient ultracentrifugation of VLPs. Supernatants from COS cells transiently transfected with pGag were concentrated through a 20% glycerol cushion and then subjected to 20-60% (w/v) sucrose density gradient ultracentrifugation. Fractions were collected from top to bottom, precipitated with 20% TCA and proteins were detected by western blot analysis. Percentage of each sucrose fraction is indicated at the bottom of each lane. Arrows indicate bands of Gag gene products (Gag $_{p55}$ and Gag $_{p41}$).

Gag_{p55} VLPs were purified from cell culture transfected with pGag to match the same Gag_{p55} gene insert expressed *in vivo*. Lastly, soluble Gag_{p55} protein was purified from yeast transfected with DNA expressing Gag_{p55}.

Expression and purification of Gag_{p55} virus-like particles

Gag proteins (Gag_{p55} and Gag_{p41}) were detected in both the supernatant and cell lysate fractions from murine cells (NIH 3T3) transiently transfected with pGag (Figure 23A). In contrast, murine cells transfected with pGag_{ΔMyr} had a higher concentration of Gag gene products in the cell lysate fraction compared to pGag, with no detectable proteins in the cell supernatants (Figure 23A). Gag_{p55} particles were purified from supernatants of COS cells (Figure 23B) transfected with pGag by ultracentrifugation (20-60% sucrose gradient) and collected fractions were analyzed for particle composition and stability by SDS-PAGE/Western blot. Gag_{p55} VLPs banded in sucrose fractions (36-44% sucrose) (Figure 23B) similar to other lentiviral particles¹⁴⁵,

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Intracellularly retained Gag proteins elicited higher titers of cellular immunity

Mice were immunized with pGag and pGag_{ΔMyr} (5 μg) at days 1, 21, and 42 (i.m.) and both humoral and cellular responses were evaluated. Mice vaccinated with pGag elicited anti-Gag_{p55} antibodies (IgG) in the sera (~1:1240 endpoint dilution titer) compared to low levels of IgG detected in mice inoculated pGag_{ΔMyr} (<1:120) (Figure 24A). In contrast, splenocytes isolated from mice vaccinated with both vaccines secreted moderate levels of INF-γ following *in vitro* stimulation with 2 overlapping peptides representing an immunodominant CTL epitope for HIV-1 Gag_{p24} (Figure 24B) or soluble Gag_{p55} protein (Figure 24C). Interestingly, splenocytes

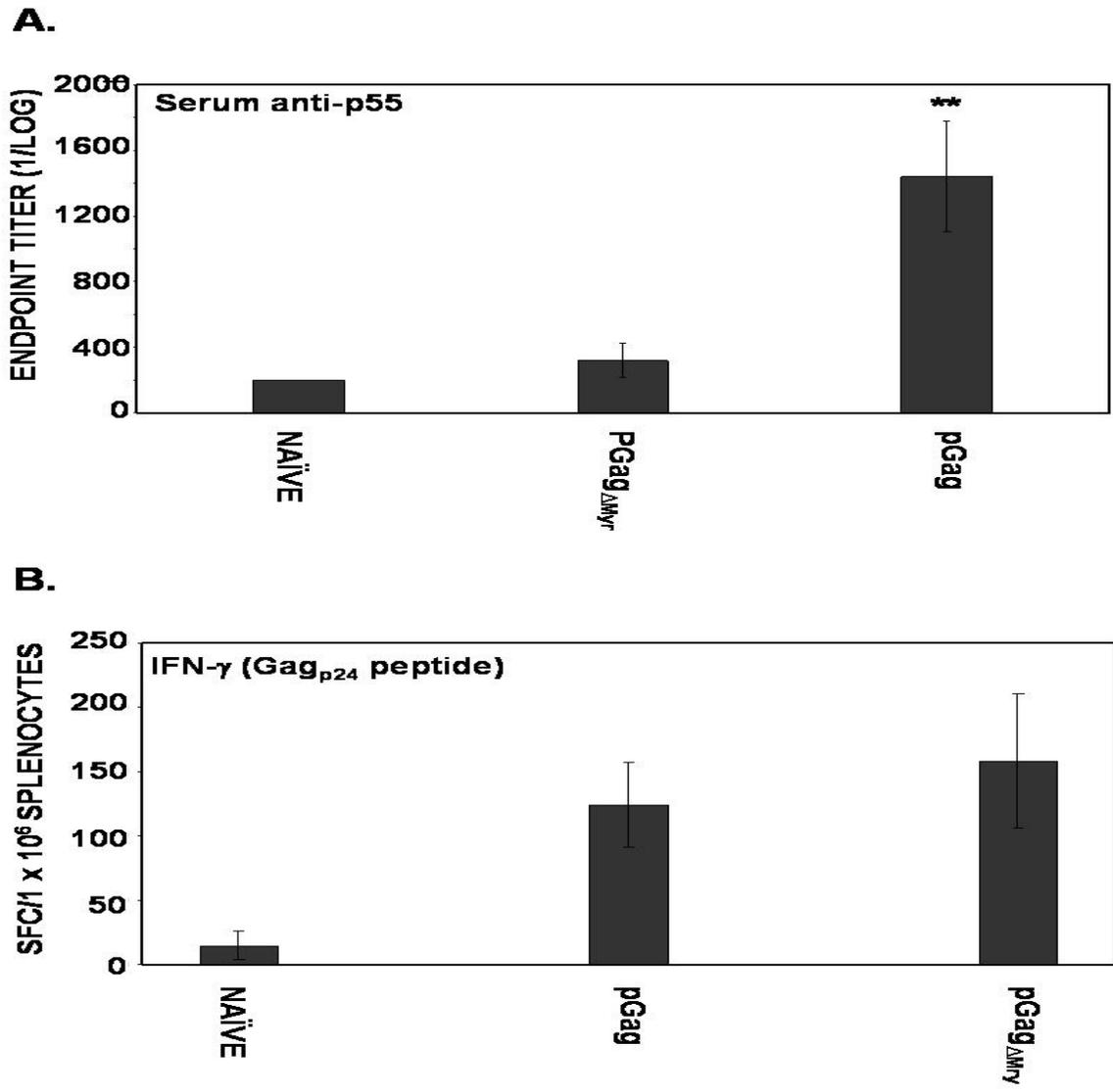


Figure 24. Immunogenicity of Gag vaccine plasmids.

Mice were vaccinated with the DNA vaccines, pGag or pGag_{ΔMYR}. Collected sera or isolated splenocytes were assayed for anti-Gag immunity (week 8). (A) Anti-Gag_{p55} antibodies were detected by ELISA, and the results are presented as the average endpoint titer. (B, C) HIV-specific IFN- γ producing splenocytes were detected by ELISPOT. Two weeks after the final boost, splenocytes were stimulated with (B) two HIV-1 Gag_{p24} peptides (1 μ g each, overlapping an immunodominant epitope) (C) or purified Gag_{p55} soluble protein (2 μ g). Cells from vector-vaccinated mice produced fewer than 10 spots per 1 X 10⁶ million splenocytes. Each bar represents the average titer of 5 mice per group plus the standard deviation minus the number of background spots. (D) Cytotoxic T-cell activity from isolated splenocytes is presented plus the standard deviation. The average percentage of cytolysis for each effector (splenocytes) to target (BC10-ME) cell ratio is shown (four independent samples, 5 mice per group). The statistical significance of the difference between groups was calculated by the Student's *t* test (** $p < 0.01$; *** $p < 0.001$ **** $p < 0.0001$).

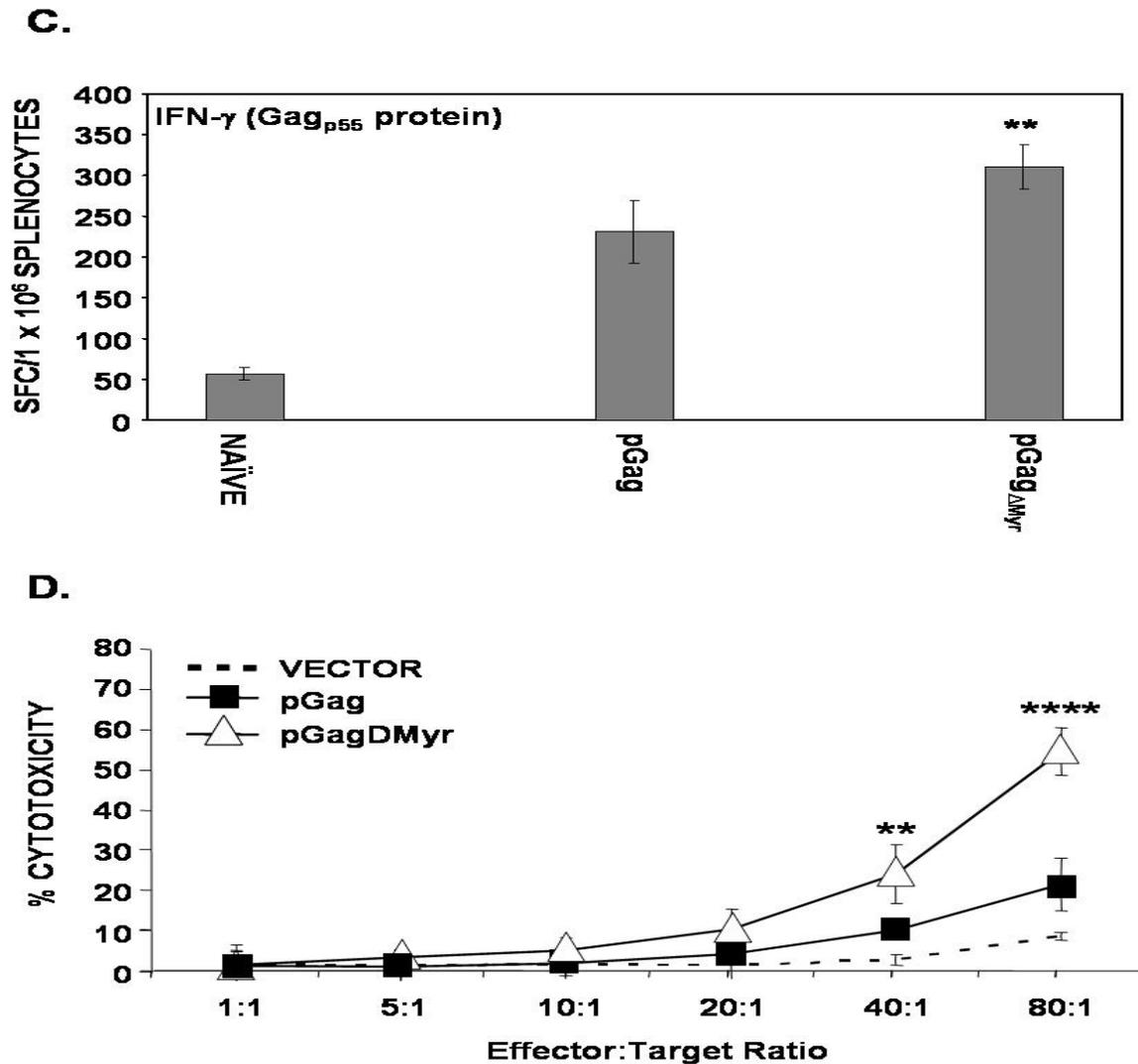


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Purified Gag_{p55} VLPs efficiently elicited both humoral and cellular immunity

from mice vaccinated with pGag_{ΔMyr} had a statistically higher number of spot forming colonies was elicited in mice immunized with pGag_{ΔMyr} compared to pGag (Figure 24C). In addition, the splenocytes from mice vaccinated with pGag_{ΔMyr} had significantly higher levels of cytotoxic activity compared to mice inoculated with pGag as measured by a nonradioactive CTL assay (Figure 24D). Therefore, DNA immunization of pGag (resulting in expression of Gag_{p55} VLPs) elicited higher titer anti-Gag_{p55} antibodies, whereas, intracellular containment of Gag proteins via DNA immunization of pGag_{ΔMyr} enhanced cellular immunity.

BALB/c mice were vaccinated with purified soluble Gag_{p55} protein (10 μg) or tissue culture purified Gag_{p55} virus-like particles (40 μg of total protein) plus CpG ODNs (10 μg) at days 1, 21 and 42 (i.m.) and both humoral and cellular responses were evaluated. Antibodies in mice immunized with both soluble Gag_{p55} protein and Gag_{p55} VLPs were detected as early as two weeks following the first vaccination and continued to rise following the third vaccination. Although mice vaccinated with soluble Gag_{p55} protein purified from yeast induced anti-Gag antibodies (1:18,000), mice immunized with purified Gag_{p55} VLPs had approximately 1 log higher titer of anti-Gag antibodies at week 8 (1:245,000) (Figure 25A). Similarly, mice vaccinated with soluble Gag_{p55} protein and Gag_{p55} VLPs elicited IFN-γ secreting splenocytes; however mice vaccinated with Gag_{p55} VLPs induced four times more INF-γ secreting splenocytes compared to mice immunized with soluble Gag_{p55} protein (Figures 25B and C). In addition, splenocytes from mice vaccinated with Gag_{p55} VLPs purified from primate cells lysed a significantly higher number of target cells compared to splenocytes isolated from mice inoculated with soluble Gag_{p55} protein (Figure 25D). Therefore, Gag_{p55} particles induced higher titers of humoral and cellular immune responses compared to soluble Gag_{p55} protein.

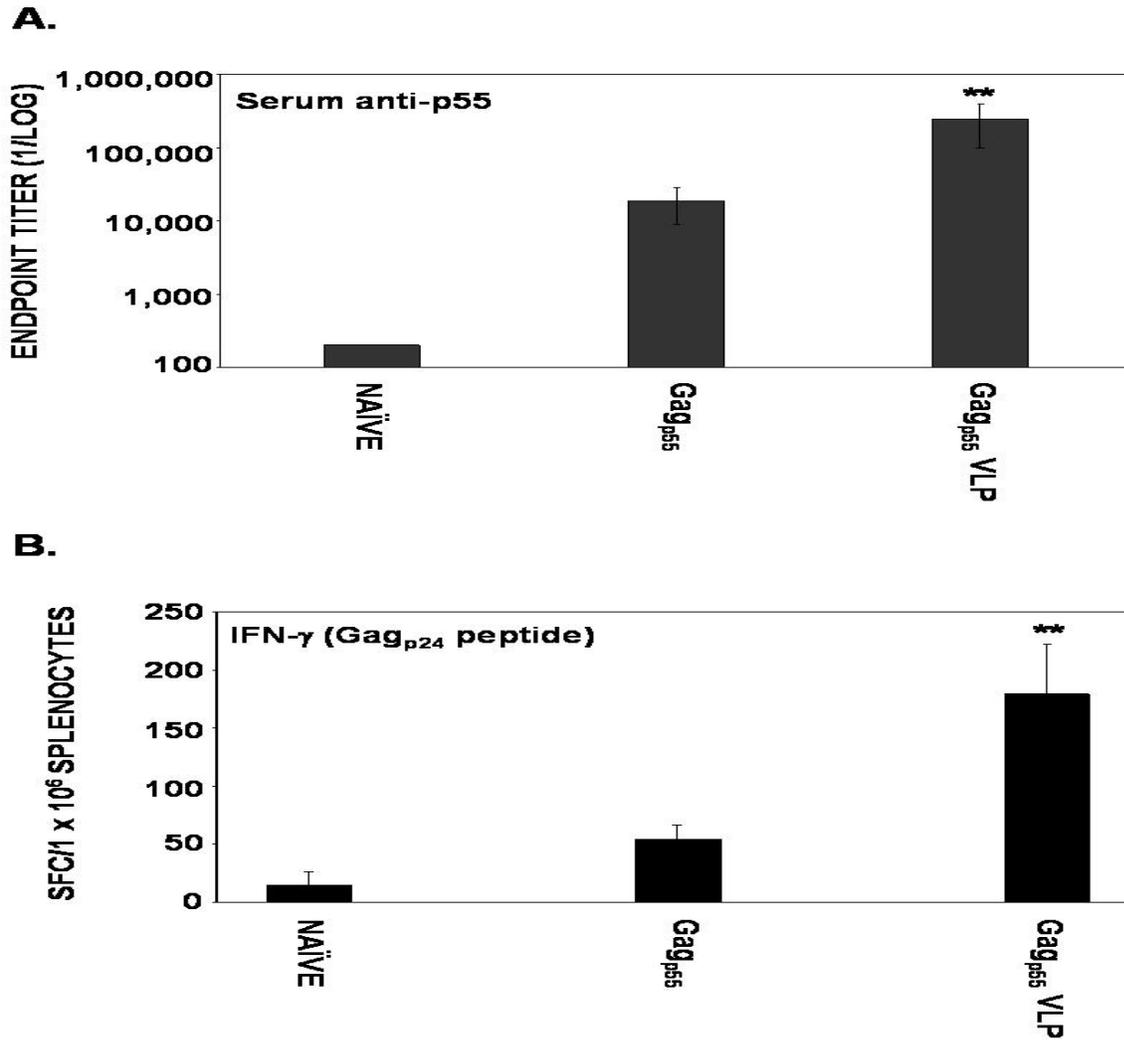


Figure 25. Immunogenicity of Gag protein vaccines.

Mice were vaccinated with purified, soluble Gag_{p55} or purified virus-like particles (VLP) co-immunized with CpG ODNs. Collected sera or isolated splenocytes were assayed for anti-Gag immunity (week 8). **(A)** Anti-Gag_{p55} antibodies were detected by ELISA, and the results are presented as the average endpoint titer. **(B, C)** HIV-specific IFN- γ producing splenocytes were detected by ELISPOT. Two weeks after the final boost, splenocytes were stimulated with **(B)** two HIV-1 Gag_{p24} peptides (1 μ g each, overlapping an immunodominant epitope) **(C)** or purified Gag_{p55} soluble protein (2 μ g). Cells from vector-vaccinated mice produced fewer than 10 spots per 1 X 10⁶ million splenocytes. Each bar represents the average titer of 5 mice per group plus the standard deviation minus the number of background spots. **(D)** Cytotoxic T-cell activity from isolated splenocytes is presented plus the standard deviation. The average percentage of cytolysis for each effector (splenocytes) to target (BC10-ME) cell ratio is shown (four independent samples, 5 mice per group). The statistical significance of the difference between groups was calculated by the Student's *t* test (* $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

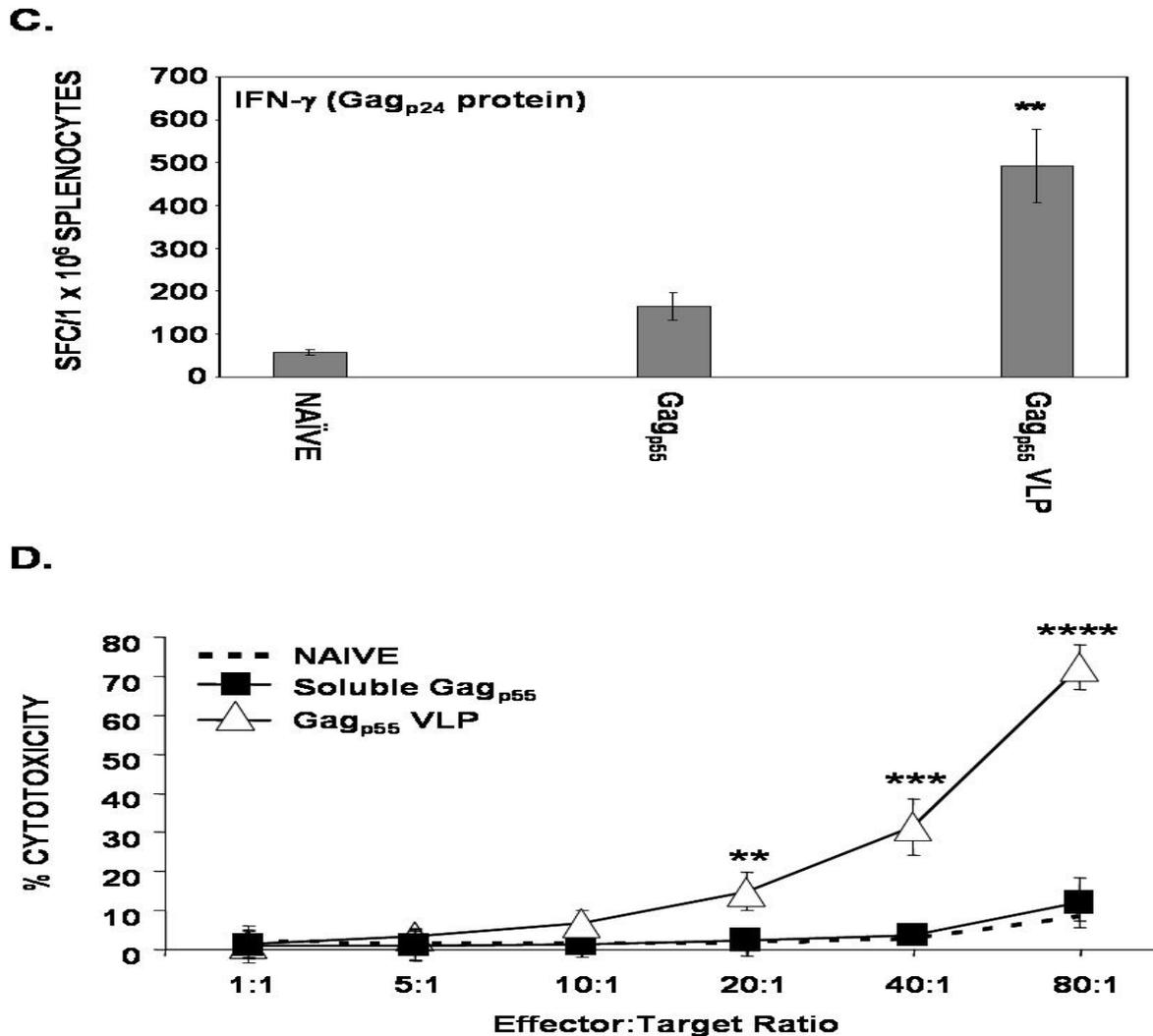


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VI.D. Discussion

Gag gene products are key components within most AIDS vaccines for the elicitation of protective immunity^{225, 287, 301, 303, 421, 471, 594, 635, 664, 665, 712}. Gag-specific T cell responses (CD4⁺ and CD8⁺) correlate with improved control of viral replication^{205, 290, 547}. In particular, anti-Gag CD8⁺ cytotoxic T cells initially control viral replication and subsequently reduce viral loads during natural infection^{55, 332, 343, 462}. These CTL responses may also prevent the establishment of persistent infection^{462, 554}. Lastly, Gag is a particularly attractive vaccine target due to cross-clade sequence conservation^{164, 690}.

In this study, mice were inoculated with four different HIV-1 Gag vaccines to compare the elicitation of immune responses by the same Gag immunogen presented to the immune system in different forms. Specifically, the type of immunity elicited by *in vivo* produced Gag proteins (pGag; secreted as particles) was compared to intracellularly retained multimers of Gag (pGag_{p55}). In a separate set of experiments, Gag virus-like particles or soluble, non-particulate Gag proteins were inoculated intramuscularly to analyze the elicited immunity. Mice vaccinated with DNA expressing Gag_{p55} VLPs (pGag) or purified Gag_{p55} VLPs elicited both anti-Gag antibodies and cellular immune responses (Figures 24 and 25). In contrast, mice vaccinated with DNA expressing Gag_{p55} with a mutated myristylation site (pGag_{ΔMyr}) elicited cellular immunity and cytotoxic activity, but had almost undetectable titers of anti-Gag antibodies (Figure 24). Mice vaccinated with soluble, non-particulate proteins had primarily humoral immune responses.

Myristylation is a key component in the particle assembly process. Gag_{p55} undergoes post-translational myristylation at the N-terminus of matrix, which allows the Gag complex to attach to the cell membrane via the myristic acid moiety^{491, 492, 496, 722, 728, 729}. Disruption of the

myristylation site (Figure 22) resulted in an accumulation of Gag proteins in the cytoplasm (Figure 23A). The higher concentration of proteins within the cytoplasm most likely increases proteosomal degradation of these polypeptides into the endogenous antigen pathway, resulting in the loading of Gag peptides on MHC class I molecules. Since, few Gag gene products were secreted from transfected cells, little anti-Gag antibodies were detected (Figure 23) similar to the immunity elicited by intracellularly retained Gag proteins using: 1) the addition of degradation signals (encephalomyocarditis virus (EMCV) 3C PR or ubiquitin), 2) mutation of the Gag-Pol frameshift site, 3) mutation of the major homology region in CA_{p24}, or 4) mutation of the myristylation site in MA_{p17}^{166, 325, 413, 600, 666, 684, 732}. However, in contrast, other reports have indicated no difference between immune response elicited by particle-forming and particle-defective vaccines⁶⁹⁵, which may be attributed to multiple factors in different model systems (dose, animal model, inoculation regimen, or immunological assays used for analysis).

In contrast, Gag_{p55} VLPs elicited robust immune responses, which were significantly higher than the immunity elicited in mice vaccinated with soluble, non-particulate Gag_{p55} protein (Figure 25). Antigen processing pathways differ for particulate antigens compared to soluble, non-particulate antigens. Professional APCs engulf and present viral peptides by a variety of mechanisms depending on the structural form of the antigens^{473, 523, 526}. These APCs process viral particles for MHC class I presentation more efficiently than soluble proteins leading to higher titer immune responses³⁴⁴. Particulate antigens, through the process of cross-presentation^{34, 226, 279, 280, 356, 513, 525, 596}, can induce an effective cell-mediated immune response in addition to a strong antibody responses. Regardless if the antigen was a soluble protein or particle, Gag antigens injected intramuscularly induced high titer anti-Gag antibodies (Figure 25A). However, these two Gag immunogens elicited marked differences in the anti-Gag splenocyte responses (as

indicated by secretion of IFN- γ) and cytolytic activity (Figure 24 and 25). Gag_{p55} VLPs elicited robust cytotoxic activity, whereas minimal cytolytic activity was detected in mice vaccinated with non-particulate, soluble Gag_{p55}. Not surprisingly, splenocytes isolated from all mice and stimulated with soluble Gag_{p55} protein had higher numbers of IFN- γ -secreting cells compared to the same cells stimulated with peptides, because the protein contains many Gag epitopes compared to the single Gag_{p24} epitope for the 2 overlapping peptides (Figure 25). In general, Gag_{p55} proteins stimulated both CD4⁺ and CD8⁺ cells, whereas the two overlapping MHC class I-restricted peptides (representing an immunodominant CTL epitope for HIV-1 Gag_{p24}) used in this study stimulated primarily CD8⁺ T cells *in vitro*⁴²³. As indicated by antibody titer, splenocyte responses, and cytolytic activity, particles composed of HIV-1 Gag gene products elicited a broader immunity than non-particulate, soluble Gag_{p55} or intracellular Gag_{p55} multimers.

Gag gene products of HIV-1 were used to study the effect of different structural forms of the same antigen on the elicitation of immune responses. In this report, Gag antigens elicited different types of anti-Gag immunity depending if these proteins were components of a virus-like particle, soluble proteins, or intracellular multimers. Overall, the expression of Gag gene products in any HIV/AIDS vaccine may elicit potentially different and more effective protective immunity depending on the structure of the proteins, the cellular compartment the antigens are located, and the antigen processing pathways used for presentation of these proteins to the immune system.

VI.E. Acknowledgments

This research was supported by National Institute of Health Grant AI51213 to T.M.R.

The authors thank Gary Nabel for providing pGag and the BC10ME cell lines. The authors would like to thank Joseph Bower and Franklin Toapanta for technical assistance and critical review of the manuscript. Also, we thank Velpandi Ayyavoo, Sharon Hillier, Ronald Montelaro, Kelly Stefano-Cole for helpful discussions. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: soluble HIV-1_{SF2} Gag_{p55} from the Chiron Corporation; HIV-1 consensus B Gag peptides from NIAID; and HIV-Ig from NABI and NHLBI.

VII. Chapter 7: Significance of Research

Overall, the virally-regulated, multi-gene VLP appears to be a promising vaccine candidate for HIV-1 although further research must determine the optimal inoculation regimen in non-human primates. The optimal vaccination regimen will include priming with a minimum of two inoculations of VLP DNA followed by two particle immunizations. Priming with DNA will ensure that all of the gene products will be expressed *in vivo*, which will result in a broadened humoral and cellular immune response. The vaccine will be inoculated by gene gun (DNA) and at the mucosa (purified particle) to enhance both mucosal and systemic immunity. Direct transfection of professional APCs, such as dendritic cells, by gene gun inoculation will increase the expression of viral proteins and VLPs *in vivo*. In addition, gene gun inoculation induces both systemic and mucosal immunity. In contrast, mucosal vaccination of DNA requires a high dose of DNA and results in a lower immune response compared to gene gun inoculation. Intramuscular inoculation may induce a strong systemic response but minimal mucosal response, which would be beneficial for a HIV vaccine. A minimum of two inoculation of DNA will be necessary to induce strong, amnestic immune responses to the viral proteins (in particular cellular responses) that are not incorporated into the virus-like particles (Tat, Rev and Vpu). Boosting with purified VLPs is necessary to enhance the neutralizing antibody response to Env.

Many of the current vaccines in the HIV Vaccine Trials Network (HVTN) express multiple viral proteins/epitopes similar to the virally-regulated, multi-gene VLP vaccine. The virally-regulated, multi-gene VLP vaccine has several advantages over these other vaccine strategies. The multi-gene, single vaccine insert is more attractive, because it allows expression of multiple proteins from a single component. The inclusion of such a number of genes in one insert is only possible due to its virally-regulated nature. Viral transcription is initiated using a single CMV promoter for efficient transcription of the VLP DNA. Numerous gene products are

produced through RNA splicing and nucleocytoplasmic transport of viral mRNAs observed in wild-type infection with HIV-1. Viral regulation through splicing results in a similar ratio of proteins observed during natural infection and prevents aberrant protein expression that may impair particle assembly or budding. The other multi-gene vaccines do not express Gag and Env from the same cell. Therefore, the VLPs produce will not incorporate Env into the viral membrane. A soluble Env immunogen (monomers or trimers) could also induce higher anti-Env antibodies, but these antibodies will not be as immunologically relevant compared to antibodies induced by the native form of Env on the surface of a particle. Unlike soluble proteins, Env incorporated into a VLP will induce neutralizing antibodies to the native structure of Env, which include conformational epitopes only found in the membrane-bound, trimeric form. Soluble Env induces much lower levels of neutralizing antibodies compared to infection with live virus even though the overall antibody titer to Env is similar. Vaccination of proteins presented to the immune system in a particulate form will activate more potent humoral and cellular responses that are rapidly restimulated upon antigen encounter especially when co-inoculated with CpG ODNs compared to soluble proteins. Vaccination with non-particulate antigens, such as the immunogens currently in the HVTN, induced a transient immune response that did not protect against infection. This difference in immune responses is most likely due to antigen uptake, processing and presentation. There are a few HIV vaccines entering human phase I trials that produce VLPs that contain Env. However, these VLPs are produced *in vitro* and can only be inoculated as purified particles. Moreover, these vaccines only express Gag and Env thus limiting the breadth of the ensuing immune response. Previously, vaccines expressing only Gag and Env have not protected the host from disease. The efficacy of the vaccine will be enhanced by including numerous viral gene products in the next generation of HIV vaccines. Humoral and

cellular immunity against the nonstructural proteins will be advantageous since these proteins are the first to be produced during natural infection. Therefore, immunity to these accessory proteins will result in the destruction of infected cells that have yet to bud infection virions thereby drastically reducing viral load. Unlike all of the vaccines currently in human trials, the virally-regulated, multi-gene VLP vaccine incorporates multiple viral proteins in their native conformation but with the versatility of single gene vaccines.

A protein/particle boost will be required to effectively enhance the neutralizing antibodies to Env. However, the purification of particles will need to be improved before entering human trials. The purification of VLPs from virally-regulated multi-gene DNA (and other systems) is expensive and time-consuming, and the particles produced only contain Gag and Env gene products. Currently, the VLPs are purified from transient transfection of virally-regulated, multi-gene plasmid DNA into mammalian cells *in vitro*. One way to increase the level of particle production is by generating stable cell lines that contain the VLP DNA. There are other purification systems, but these approaches have multiple issues including anti-cellular immunity, non-human glycosylation of Env and lower particle yield. Particles produced by any of the purification processes may induce anti-self immunity due to cellular proteins that are incorporated into the VLP during the purification process. The immunity to cellular proteins should be reduced in a primate system, because cell lines from the same species can be used for *in vitro* purification unlike the rodent system which uses a monkey cell line for particle purification. However, a protein/particle component will need to be included to enhance antibodies to Env based on other studies. The co-inoculation of CpG ODNs with VLPs will greatly enhance the immunity to HIV.

The first study that should be completed in non-human primates is to compare the virally-regulated, multi-gene VLP vaccine to codon-optimized single genes that do (gag and env expressed from same cell) or do not form particles (gag and env expressed from different cells) using the same viral protein sequences and inoculation regimen (DNA-DNA-VLP-VLP). The vaccine inoculums will have to be given at equal concentrations and also normalized for protein expression *in vitro* to compensate for the lower protein expression observed with the virally-regulated VLP DNA. Immune function (ability to kill infected cells or prevent infection of susceptible cells) rather than total response (non-neutralizing antibody or secretion of cytokines) will resolve which vaccine is the best and will be determined based on several criteria: induction of broad humoral and cellular responses (number of epitopes not level of activation), neutralization capacity of antibodies (systemic and mucosal), cytotoxic capability of CTLs (systemic and mucosal), and most importantly, protection against viral challenge. If none of the vaccines induce protection in the non-human primates, the efficacy of the vaccines will be determined by immune functions previously stated in addition to viral load, setpoint, CD4 count and disease progression. Although protection from infection is the ultimate goal of vaccination, it may be beneficial to characterize the immune response elicited that may protect from disease or prolong the asymptomatic stage of infection. Many of the immune parameters that are currently being used to measure the immune response elicited against HIV may not correlate with protection. However, a vaccine expressing multiple proteins may elicit immunity that will control viral replication by eliminating infected cells prior to budding of infectious virions. This analysis of the immune response will determine whether the current strategies in humans and non-human primates are ideal or alternative strategies should be considered. A more

comprehensive evaluation of the immune response will be completed to characterize the correlates of protection if the VLPs prove to be efficacious in nonhuman primates.

Further research in non-human primates will be needed to enhance particle production (stable cell lines, inactivation of PR and deletion of *pol*) and minimize the contribution of cellular immunity (species-specific cell lines). Also in non-human primates, methods to enhance broadly cross-reactive neutralizing antibodies to Env by sequence/structure modifications (de/hyper-glycosylation, variable loop deletions, truncation, multi-strain/clade or consensus sequences) will also need to be examined. Multiple generations of the VLP vaccine will need to be tested *in vitro* and in a rodent/small animal system. However, only a small number of these vaccines will proceed to nonhuman primate immunogenicity studies based on their ability to induce the killing of infected cells and prevent HIV from infecting susceptible cells. Once the optimal vaccine candidate and regimen is determined in non-human primates, safety and immunogenicity of the VLP vaccine can be tested in a Phase I human trial.

Assuming success in non-human primates, the virally-regulated, multi-gene VLP vaccine should induce both humoral and cellular responses to a variety of viral proteins in humans. Both humoral and cellular immunity should be elicited using a combinatorial vaccine regimen (two DNA, two purified VLPs) (Figure 29). This immune response should include broadly cross-reactive neutralizing antibodies and cellular immunity to multiple viral proteins. Once the vaccinated individual becomes infected with HIV-1 (HIV infection of unvaccinated individuals, see Figure 3), strong amnestic (memory) humoral and cellular responses will be induced at the mucosa and systemic levels. APCs (dendritic cells and macrophages) will produce the proteins *in vivo*, and particles will bud from the transfected cells following VLP DNA immunization. These cells will become activated and recruit other immune cells. Systemic and mucosal

immune responses will be elicited against multiple viral proteins following two DNA vaccinations and will be greatly enhanced following two immunizations with purified VLPs. The purified VLPs/CpG ODNs will be inoculated in the nasal passage, enter the NALT and restimulate HIV-specific immune cells induced by priming with DNA. The presence of CpG ODNs will activate dendritic cells, macrophages and B cells. NK and T cells will also be activated indirectly. The highly concentrated area of cells will undergo clonal expansion and result in a very robust local immune response. These activated, HIV-specific immune cells will then migrate to the lymph system and disperse throughout the body. Neutralizing and opsinizing antibodies will prevent further infection of susceptible cells, while infected cells will be destroyed by ADCC and CTLs. A drop in the number of CD4+ T cells will not be seen due to the increase in vaccine-induced, HIV-specific CD4+ T cells and controlled level of replication. A rapid but potent amnestic response will control or clear the virus from the body before the virus can establish a persistence infection. Viral escape mutants will be less likely to arise shortly after infection because the immune response elicited by the VLP vaccine will encompass many different epitopes in multiple viral proteins. Viral replication will be rapidly controlled (by destruction of infected cells before virion release and neutralizing antibodies) and will lead to a very low viral setpoint or the virus will be cleared. There are two possible outcomes following infection: 1) The immune system will clear the virus, and the patient will remain symptom/disease free (sterilizing immunity). 2) The immune system will control but not clear the virus (very low viral set point), and the patient will remain infected but will have a very slow disease progression similar to a LTNP or will eventually die from a non-HIV cause. If the individual remains infected, the generation of viral escape mutants will be similar to that

A.

	DNA (gg)	DNA (gg)	Purified particle (IN)	Purified particle (IN)
Non-infected individual following VLP vaccination	Humoral – all proteins Cellular – all proteins Systemic – yes Mucosal – yes	Humoral – all proteins Cellular – all proteins Systemic – yes Mucosal – yes	Humoral – Gag/Pol/Env Cellular – Gag/Pol/Env Systemic – yes Mucosal – yes	Humoral –Gag/Pol/Env Cellular – Gag/Pol/Env Systemic – yes Mucosal – yes

B.

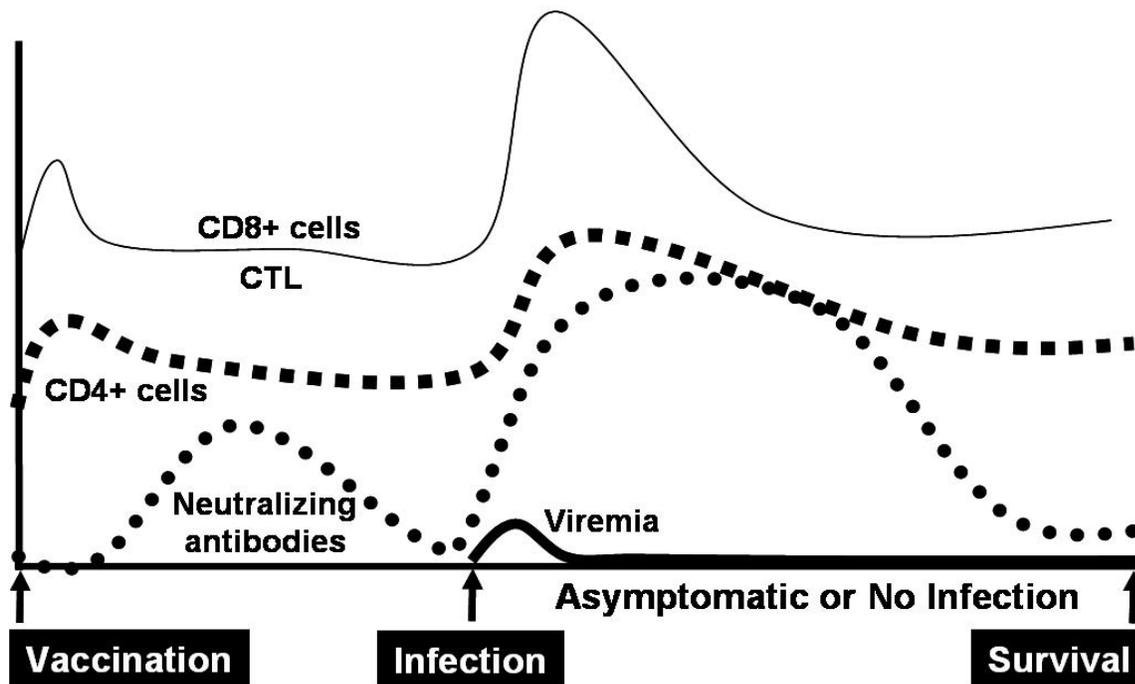


Figure 26. Immune response in VLP-vaccinated individuals infected with HIV.

(A) Initially, an individual is vaccinated with the VLP vaccine. Anti-HIV cellular and humoral immune responses are elicited following multiple vaccinations (two DNA and two purified particle inoculums) at mucosal and systemic compartments. (B) Following vaccination, the elicited immunity HIV decreases to background levels. The patient is then infected with HIV-1, and amnestic humoral and cellular responses are recalled to control the initial viremia. CTLs and ADCC will remove infected cells, while neutralizing and opsonizing antibodies remove cell-free virus. The multi-faceted immune response clears the virus from the body or decreases viral loads to undetectable levels. Following the clearance or containment of the virus, the patient will be virus-free or will be infected, but asymptomatic. Even in the case of persistent infection, the patient will remain asymptomatic/disease free for many years similar to that of LTNPs.

observed in LTNPs because the viral setpoint in the vaccinated individuals will be very low or undetectable.

In contrast to the immune response elicited to the virally-regulated, multi-gene VLP vaccine, immunization with single gene vaccines that generate VLPs +/- Env will not protect the individual from disease. Single gene vaccines expressing VLPs containing Gag only and Env as a soluble protein (separately) will not induce anti-Env antibodies capable of neutralizing the virus once the individual becomes infected. Cellular responses may be diminished following the Gag only particle inoculations, because the particle will not be able to enter susceptible cells in an Env-mediated manner. Each gene will be inoculated as separate plasmids, which will increase the cost and complexity of vaccine production. Single gene vaccine strategies compose the majority of the vaccines currently in non-human primate or human trials and have not elicited protection from infection. The virally-regulated, multi-gene vaccine has numerous characteristics that set it apart from these other vaccine strategies. The virally-regulated nature allows the VLP vaccine to contain numerous viral proteins expressed similar to natural infection (order and quantity of protein expression and native conformation). More specifically, the only target of neutralizing antibodies, Env, will be in the native conformation similar to that of wild-type virus. This may result in antibodies that are more immunologically relevant, which are directed at conformation-dependent epitopes. VLPs containing Env are advantageous, because they can penetrate restricted immune compartments that soluble proteins cannot enter such as the associated lymphoid tissue through M-like cells. Particulate antigens include both humoral and cellular immune responses and have intrinsic properties that activate APCs and promote maturation/long-term immunity.

Other VLP vaccines that produce VLPs containing Gag and Env from codon-optimized genes have numerous issues. First and foremost, this vaccination approach only contains Gag and Env, so the likelihood of viral escape mutants dramatically increases with this type of vaccine. This vaccine will have to be used in conjunction with other vaccine strategies to include other viral proteins to increase the breadth of the immune response. Also, this vaccine approach does not have the versatility of single genes that do not produce particles containing Env or the virally-regulated, multi-gene VLP vaccine. Lastly, this particle production system overexpresses Gag and Env. The Env incorporated into the VLP will be more unstable due to an abnormally enhanced number of Env molecules on the surface of the particle, and many of these trimers will be composed of uncleaved Env_{gp160}. Overexpression of Env has been shown to be detrimental for particle production and Env incorporation using VLP production by stable cell lines expressing codon optimized HIV-1 Gag_{p55} and Env_{gp160}. In either case, the antibodies produced to these artificial Env trimers will not neutralize virus containing the native form of Env seen during natural infection.

There are many different categories of individuals that will need to be vaccinated against HIV: individuals that are uninfected or infected with HIV-1, elderly, infants and immunocompromised. Although it will work more efficiently as a prophylactic vaccine, the VLP vaccine can be used as a therapeutic treatment for infected individuals or in immunocompromised patients (elderly, infants, individuals with cancer and transplant recipients, etc.). However, different vaccination regimens could be employed to induce the optimal immune response in these individuals (i.e. higher concentration of inoculum, additional inoculations, combination of other adjuvants, etc.). In HIV-infected individuals, the VLP vaccine will minimize the level of viral replication and prolong the asymptomatic phase. One aspect of the

VLP vaccine that may help with the majority of these individuals is the CD4-independent elicitation of immune responses by co-vaccination of CpG ODNs. After the virally-regulated VLP vaccine has been demonstrated to induce protective responses in healthy individuals, more research will have to focus on the induction of protective immunity in the other groups. Most likely, the individuals will require multiple immunizations throughout their life to maintain effective immunity. Unlike vaccination with a live-attenuated virus, the use of multiple inoculations over the lifetime of the host is common among vaccines that do not persist in the host (i.e. DNA and protein subunit vaccines). It is highly unlikely that a single (or even two) dose vaccine regimen will elicit protective immunity for the lifetime of the host especially considering the mutation rate of the virus for any vaccine/regimen to HIV-1. That is why the proposed vaccine includes two DNA inoculations followed by two particle vaccinations. Protective immunity elicited by a vaccine today may not protect against the HIV strains circulating in the human population in twenty years. However, the VLP vaccine can be modified to meet the ever-changing face of HIV similar to the influenza vaccine. The viral sequences from most prevalent strains circulating in the population at the time can be engineered into the VLP vaccine with relative ease. In conclusion, the virally-regulated multi-gene VLP vaccine is a novel strategy that will induce broadly, cross-reactive immunity to several proteins, and in particular humoral responses to native, immunologically relevant epitopes in Env, that should protect individuals from infection with HIV-1.

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Young KR, Ross TM
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Sincerely,
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16 March 2005

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