HIV-1/SIV NEUTRALIZING ANTIBODY GENE DELIVERY:
A NOVEL VACCINATION APPROACH

DISSERTATION

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By

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*****

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ABSTRACT

The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). Since the start of the pandemic 26 years ago, nearly 32 million people have died of AIDS. More than 30 million individuals are currently HIV/AIDS carriers, with nearly 7,500 people becoming infected each day. Due to the lack of a cure of AIDS currently, creating an efficacious, safe and affordable vaccine has become our top priority to control this pandemic.

An ideal HIV vaccine would be an immunogen that elicits antibodies which can broadly neutralize field isolates of HIV-1. Unfortunately, such an antigen has not been identified to date. Many HIV-1 vaccine candidates have stimulated cell-mediated immune responses that control viral replication without preventing infection. However, passive immunization studies demonstrated that HIV-1 infection could be prevented in rhesus monkey models if sufficient levels of one or several broadly neutralizing antibodies (NAbS) are present in the animal’s serum prior to infection. The challenge is how to deliver these broadly NAbS as a prophylactic vaccine to millions of people.

Our approach is predicated on the ability to achieve sustained, high concentrations of neutralizing antibodies in the systemic circulation by antibody gene transfer. The stable delivery of pre-selected, broadly NAb genes will provide protection against pathogenic simian human
immunodeficiency virus/simian immunodeficiency virus (SHIV/SIV) challenge. Using this approach, effective humoral immunity can be supplied to the host to prevent or significantly blunt viral infection. Towards this goal, our laboratory previously demonstrated long-term expression of an anti-HIV-1 antibody (IgG1b12) in Rag-1 mice following recombinant adeno-associated virus serotype 2 (rAAV2) mediated delivery of heavy and light chain genes into mouse skeletal muscle. Unfortunately, the expression level was below that considered to be efficacious.

To improve the serum antibody expression levels following muscle mediated gene transfer, we first used the single-chain variable-fragment X5 (scFv X5) which is smaller than IgG1b12 and can be expressed by a single promoter. We also utilized AAV serotype 1 that has been demonstrated to efficiently mediate gene delivery to muscle. Additionally, we identified an optimized leader peptide (SL1) that improved antibody secretion efficiency. By utilizing a self-complementary (SC) rAAV1 vector, we improved the scFv X5 expression compared to standard single stranded (SS) rAAV1 vector by 3 to 14 fold. To address whether the dose is scalable from mice to primates, we performed a dosing study and showed that dosing on a per kg basis is scalable. However, the levels of circulating scFv X5 in rhesus macaques were unable to prevent a challenge virus infection.

Due to the short half-life of scFv X5, we looked another scFv antibody 3B3 and developed novel fusion constructs based on the constant region hinge-CH2-CH3 (hCC) of IgG1 to increase half-life. These genetic fusions increased serum levels by 100-1,000 fold. rAAV8 was demonstrated to mediate 2-fold higher transgene expression and the woodchuck hepatitis post-transcriptional regulatory element (WPRE) was shown to further increase levels 4 - 6 fold. Two strong ubiquitous promoters (cytomegalovirus [CMV] and CMV-chicken beta-actin hybrid [CAG])
showed equal strength in expressing the transgene in muscle tissue. These improvements resulted in circulating levels of transgene exceeding 600 μg/ml in mice and up to 43.0 μg/ml in the serum of rhesus macaques. However, challenge studies using a SHIV162P3 via the intra-rectal route failed to achieve protection in vaccinated macaques. Pre-existing neutralizing antibodies to AAV capsid may have had a role in the failure to protect.

Finally, we explored two rhesus-derived scFv SIV NAbs (4L6 and 5L7) and one CD4 based inhibitor (N4) consisting of part of the rhesus CD4 sequence. These three molecules were fused with a rhesus IgG2 hCC. In total, nine rhesus monkeys were injected with rAAV1 containing 4L6, 5L7, or N4 (three animals per group). Overall, we achieved significant improvement in transgene expression by using this approach. Long-lasting high level serum expression and neutralizing activities were achieved in 8 of the 9 monkeys. More importantly, six out of nine vaccinated monkeys were completely protected against a virulent SIV challenge through the intravenous (IV) route. One infected monkey had delayed viremia by two weeks and all three infected monkeys had significantly lower viral set points and viral loads compared to challenged naïve control monkeys. Further analysis of their sera revealed that these three infected monkeys produced anti-antibodies against the scFv domain of 5L7, the conserved Fc fragment, or the CD4 D1D2 domains, respectively. Therefore, blockage of neutralizing activity by anti-antibodies is most likely responsible for these three animal’s infection.

As a proof-of-concept experiment, we demonstrated the feasibility of using rAAV-mediated gene transfer into muscle tissue to achieve sufficient levels of circulating neutralizing antibodies to block virus infection and lower viral loads in the infected animals. This “reverse immunization” strategy bypasses the adaptive immune system and holds significant promise as a designer
approach for an effective HIV vaccine. Considering the diversity of HIV-1, the success of our approach is predicted on the availability of potent and broadly NAbs and inhibitors. The impact of humoral immunity targeting the vector and transgene was demonstrated and has the potential to adversely affect our vaccination approach. In addition, this novel strategy can be applied not only as a prophylactic HIV vaccine, but also as a therapeutic vaccine against other infectious diseases and cancers.
Dedicated to people infected with HIV
ACKNOWLEDGMENTS

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PUBLICATIONS


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<td>$\alpha$</td>
<td>Alpha</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Beta</td>
</tr>
<tr>
<td>A1AT</td>
<td>Alpha1-antitrypsin</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADE</td>
<td>Antibody-dependent enhancement</td>
</tr>
<tr>
<td>AID$_{50}$</td>
<td>50% animal infectious dose</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline-phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
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<tr>
<td>bCG</td>
<td>Beta subunit of choriogonadotropic hormone</td>
</tr>
<tr>
<td>bDNA</td>
<td>Branched DNA</td>
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<tr>
<td>CAG</td>
<td>Cytomegalovirus-chicken beta-actin hybrid</td>
</tr>
<tr>
<td>CCS</td>
<td>Cosmic calf serum</td>
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<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<td>CD4BS</td>
<td>CD4 receptor-binding site</td>
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<td>CD4i</td>
<td>CD4 inducible epitope</td>
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CDC Complement-dependent cytotoxicity
cF.IX Canine factor IX
CIP Ciprofloxacin Hydrochloride
CMV Cytomegalovirus
CMI Cell-mediated immunity
CP Cell pellet
CPE Cytopathic effect
CTL Cytotoxic T lymphocyte
CVs Column volumes
DC Dendritic cells
°C Degrees celsius
DNA-PK DNA-dependent protein kinase
DRP DNase resistant particle
DSC Differential scanning calorimetry
Env Envelope
Epo Erythropoietin
Fab Fragment of antigen binding
Fc Crystallizable fragment
FcγR IgG Fc receptor
FcRn Neonatal Fc receptor
FDC Follicular dendritic cell
F.IX Factor IX
γ Gamma
g Gram(s)
$g$ Relative centrifuge force

GALT Gut-associated lymphoid tissue

HAART Highly active anti-retroviral therapy

HBV Hepatitis B virus

hF.IX Human factor IX

IC$_{50}$ Concentration of a drug needed to inhibit viral replication by 50%

IC$_{90}$ Concentration of a drug needed to inhibit viral replication by 90%

hCC Hinge-CH2-CH3 domians of IgG

HPV Human papillomavirus

HVR Hypervariable loop regions

IAVI International aids vaccine initiative

ID$_{50}$ A pathogen that produces infections in 50% of the population

HIV Human immunodeficiency virus

HIV-1 Human immunodeficiency virus type 1

HIV-2 Human immunodeficiency virus type 2

HLA Human leukocyte antigen

HSPG Heparin sulfate proteoglycan

HVR Hypervariable region

IM Intramuscular

IN Integrase

ITR Inverted terminal repeat

IV Intravenous

IVIG Human intravenous immunoglobulin IgG

k Kilo
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<td>LAL</td>
<td>Limulus amoebocyte lysate</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>LTRs</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>M</td>
<td>Moles per liter</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MDP</td>
<td>Threonyl muramyl dipeptide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MHC I</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MID50</td>
<td>The 50% minimal infective dose</td>
</tr>
<tr>
<td>Min.</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NAbs</td>
<td>Neutralizing antibodies</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primate</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NL</td>
<td>Natural IgG leader sequence</td>
</tr>
<tr>
<td>OD450nm</td>
<td>Optical density at 450nm</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypurine tract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>Q-RT-PCR</td>
<td>Quantitative real-time reverse transcription PCR</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant adeno-associated virus</td>
</tr>
<tr>
<td>rAd5</td>
<td>Recombinant human adenovirus type 5</td>
</tr>
<tr>
<td>RBS</td>
<td>Rep binding site</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SBBC</td>
<td>Sydney blood bank cohort</td>
</tr>
<tr>
<td>SC</td>
<td>Self-complementary</td>
</tr>
<tr>
<td>sCD4</td>
<td>Soluble CD4</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain variable-fragment</td>
</tr>
<tr>
<td>SC rAAV</td>
<td>Self-complementary rAAV vector</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted alkaline phosphatase</td>
</tr>
<tr>
<td>SEC</td>
<td>Hepatic sinusoid endothelial cell</td>
</tr>
<tr>
<td>SHIV</td>
<td>Simian human immunodeficiency virus</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SIVmac</td>
<td>SIV from <em>Macaca mulatta</em></td>
</tr>
<tr>
<td>SL1</td>
<td>Synthetic leader peptide 1</td>
</tr>
<tr>
<td>SL2</td>
<td>Synthetic leader peptide 2</td>
</tr>
<tr>
<td>SPC</td>
<td>Spots forming cells</td>
</tr>
<tr>
<td>SPs</td>
<td>Signal peptides</td>
</tr>
<tr>
<td>S-S</td>
<td>Disulfide</td>
</tr>
<tr>
<td>SS rAAV</td>
<td>Single-stranded rAAV vector</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans-activation response element</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tms</td>
<td>Melting temperatures</td>
</tr>
<tr>
<td>T-PEB</td>
<td>Tissue specific protein extraction buffer</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>TRS</td>
<td>Terminal resolution site</td>
</tr>
<tr>
<td>V</td>
<td>Volt(s)</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VP1</td>
<td>Viral capsid protein 1</td>
</tr>
<tr>
<td>VP2</td>
<td>Viral capsid protein 2</td>
</tr>
<tr>
<td>VP3</td>
<td>Viral capsid protein 3</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VVC</td>
<td>Viral core laboratory at the Research Institute at Nationwide Children’s Hospital</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>wtAAV</td>
<td>Wild-type adeno-associated virus</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Overview

1.1.1 HIV-1 pandemic

The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) (Gallo, Salahuddin et al. 1984). It was first identified by Drs. Luc Montagnier and Francoise Barre-Sinoussi at the Pasteur Institute in 1983, for which they shared the 2008 Nobel Prize in medicine (Barre-Sinoussi 1983). HIV exists as two strains, type 1 (HIV-1) and type 2 (HIV-2). HIV-1 accounts for the vast majority of AIDS cases, while HIV-2 is far less pathogenic. Therefore, most investigations have been focused on HIV-1 (Popper, 1999). HIV-1 is further classified into three groups: 1) the “Major” group M; 2) the “Outlier” group O; and 3) the “New” group N. These three groups represent putative introductions from three different geographical locations of simian immunodeficiency virus (SIV) into humans. Group O is restricted to west-central Africa and group N was discovered in the west coast of Africa in 1998. More than 90% of HIV-1 belongs to group M in which at least 9 genetically distinct subtypes (or clades) including A, B, C, D, F, G, H, J, and K were discovered.
HIV-1 is transmitted by sexual contact, blood transfusion, or HIV-positive mothers to babies during pregnancy, birthing process, or afterwards from breastfeeding. HIV infection is characterized by the depletion of CD4+ helper T-lymphocytes and monocytes/macrophages, which leads to severe immunosuppression, neurological disease, and opportunistic infections (Fauci, Lane et al. 1998). Since the start of the pandemic, nearly 32 million people have died of AIDS in the world, with 8,200 people dying daily. More than 30 million individuals are currently HIV/AIDS carriers, with nearly 14,000 to 16,000 people becoming infected each day. Ninety-five percent of the new infections take place in the developing world. Due to the lack of an effective cure of AIDS currently, creating an efficacious, safe and affordable vaccine is the best approach to control this virus.

1.1.2 Molecular structure of HIV-1

HIV-1 is a lentivirus that consists of two identical 9.5 kb strands of RNA. The RNA genomes are packaged within a core (or capsid) of viral proteins and surrounded by a phospholipid bilayer envelope derived from the host cell membrane (Abbas, 2000) (Figure 1.1). Beneath the viral envelope layer lies the matrix which is mainly composed of protein p17. Inside the matrix shell is the viral capsid core, which is usually bullet-shaped and consists primarily of the protein p24. The two strands of RNA and three enzymes reverse transcriptase (RT), integrase (IN), and protease (PR) are located within the virion core. In the early stages of HIV-1 infection, the viral RNA is converted into double-stranded linear DNA by viral RT. IN catalyzes viral and host DNA cleavages and recombination resulting in covalent linkage of linear double-stranded viral DNA into the host genome (Goff 1992). PR cleaves newly synthesized polyproteins at the appropriate sites to create the mature protein components for infectious HIV virus particles.
The HIV-1 DNA genome contains three major coding regions: 1) *gag* (core); 2) *pol*; and 3) *env* (gp160) (Figure 1.2). The *gag* gene encodes the core structural proteins including p24 and p17. The *env* gene encodes the envelope glycoproteins gp120 and gp41, which make up the viral spikes on the viral membrane surface. Each virus particle usually has about 72 of these spikes (Gelderblom, Hausmann et al. 1987). The *pol* gene encodes RT, IN, and PR enzymes required for viral replication. The viral RT is an error-prone enzyme, which generates significant levels of sequence variance. Possible error mechanisms in general are: 1) direct incorporation of a non-complementary nucleotide which generates a single-base substitution; 2) slippage of the two DNA strands that may occur at repetitive sequences to generate either a deletion in the template strand or an addition in the primer strand at one or more positions; and 3) misincorporation followed by misalignment of the template-prime which may cause frame shifts. These events can yield not only single-nucleotide mutations but also may operate over large distances to generate mutations involving many nucleotides. The error rate by HIV-1 RT ranges from 1 bp per 1.7 to 4 kb, which is higher than RT from the other retroviruses and much higher than mammalian cellular DNA polymerase (Battula and Loeb 1974; Bebenek, Abbotts et al. 1989; Roberts, Preston et al. 1989). Because of the error prone RT, the *in vivo* error rate per replication cycle for the 9.5 kb HIV-1 genome is estimated to be one to three misincorporations. This high mutation rate results in the variation of the virus so that it is able to evade host immune responses and drug therapies. Resistant mutations can be selected under these conditions producing “escape” phenotypes.

Six other regulatory genes (*tat*, *rev*, *vif*, *nef*, *vpr*, and *vpu*) modulate viral production and pathogenesis in *trans* through the corresponding products (Tat, Rev, Vif, Nef, Vpr, and Vpu). Tat (trans-activator of transcription) is an important regulator. Tat binds to a special stem-looped sequence called trans-activation response element (TAR) at the 5’-end of viral transcripts. The
interaction of Tat with TAR increases the levels of transcription initiation and elongation. In another words, when Tat is absent, the initiation directed by long terminal repeat (LTR) is slow and the transcriptional elongation is inefficient. Rev (regulator of virion) facilitates the transportation of HIV mRNA transcripts from the nucleus to the cytoplasm, where the HIV structural proteins and RNA genome can be assembled. Vif (viral infectivity factor) is essential for viral infection and replication. In the absence of Vif, cellular host protein APOBEC3G (A3G) will cause hypermutation of the viral genome resulting in non-infectious virons. Vif inhibits A3G from entering the virion during virus budding by inducing its degradation through the ubiquitin-proteasome pathway (Sheehy, Gaddis et al. 2003; Yu, Yu et al. 2003). More recently, site directed mutagenesis studies have demonstrated that the Vif PPLP motif (residues 161 - 164) is responsible for binding of A3G to a Cullin5-ElonginBC E3 ubiquitin ligase complex leading to proteasomal degradation of A3G (Donahue, Vetter et al. 2008). Vpr (viral protein R), a small 15 kD protein, accumulates at late times in infection. Vpr promotes nuclear localization of the viral pre-integration complex and is required for viral replication in non-dividing cells such as macrophages (Agostini, Popov et al. 2002). It also induces cell cycle arrest in the G2 phase by preventing activation of the p34cdc2/cyclin B complex, which is required for host cell entry into M phase (He, Choe et al. 1995; Jowett, Planelles et al. 1995). By inhibiting p34cdc2/cyclin B complex activation, Vpr may delay or prevent apoptosis of HIV infected cells that would act to decrease the level of virus production.

Vpu (viral protein U), a membrane-spanning phosphoprotein, is only found in HIV-1. Vpu promotes viral particles to release from infected cells. Vpu down regulates CD4 expression by targeting it to the proteasomal degradation in the ER. Therefore, the intracellular half-life of CD4 is reduced from 6 hours to 12 minutes in infected cells. One direct consequence of this is to
facilitate transportation and processing of gp160. CD4 can otherwise capture envelope protein within the ER to form gp160-CD4 complexes. Thus, Vpu could potentially reduce the formation of the gp160-CD4 complex by decreasing the intracellular CD4 concentration and thereby increasing the availability of gp160. Therefore, in HIV-1-infected cells, Vpu ensures sufficient quantity of Env protein for incorporation into mature virions, which improves viral production efficiency.

Nef, initially believed to be a negative regulatory factor, seems to be a major factor responsible for maintaining high viral loads. Nef promotes the survival of infected cells by down-regulating the expression of several important surface molecules such as CD4 and MHC-I by endocytosis (Schwartz, Marechal et al. 1996). This down-regulation possibly interferes with cellular communication and results in loss of lytic functions in cytotoxic T cells (Lee, Saksela et al. 1996; Schwartz, Marechal et al. 1996). Nef also interacts with the host cell’s signal-regulating kinase 1 (ASK1) to keep infected cells alive longer, while killing nearby non-infected Fas-expressing cells by Fas and Fas ligand (FasL) mediated apoptosis (Geleziunas, Xu et al. 2001). Viruses expressing fully functional Nef proteins have a strong growth advantage in vivo (Whatmore, Cook et al. 1995).

The viral DNA also contains two long terminal repeats (LTRs) at each end. LTRs regulate viral integration into the host genome, viral gene expression, and viral replication in cis. The LTR is further divided into three regions in the order of 5’-U3-R-U5-3’ (Laprevotte, Pupin et al. 2001). U3 and U5 are the unique regulatory sequences at the 3’ and 5’ ends of the LTR respectively; R corresponds to the terminal repeat RNA. In the upstream 3’ LTR, a polypurine tract (PPT) and a central PPT (cPPT) serve as two primers generated by RNase H digestion of viral RNA strand for
DNA plus-strand synthesis by RT. Similarly, downstream of the 5’ LTR, a primer-binding site (PBS) is responsible for minus-strand reverse transcription. Host RNase H facilitates the synthesis of the second strand viral DNA by degrading the RNA strand in the DNA/RNA hybrid.

1.1.3 HIV-1 pathogenesis

The viral life cycle begins with the binding of gp120 to the target cell via its primary receptor CD4, which induces a conformational change in the viral envelope subunit of gp120 and allows gp120 binding to chemokine co-receptors CCR5 or CXCR4. Other co-receptors such as CCR1, CCR2b, CCR3, and CD8 have also been found (Alkhatib, Berger et al. 1997; Dittmar, Zekeng et al. 1999; Saha, Zhang et al. 2001). The conformational change of gp120 also results in the exposure of a previously buried CD4-inducible epitope (CD4i) on the envelope surface. Fusion of the viral lipid envelope into the cellular membrane occurs through the fusion domain of gp41. Engagement of receptor CD4 and co-receptor CCR5 or CXCR4 with gp120 causes a conformational change in gp41, which exposes a hydrophobic region named the fusion peptide. The insertion of this peptide into the cell membrane promotes viral/cellular membrane fusion, so that the HIV genome can then be passed into the target cell (Figure 1.3).

Crystallographic analysis of the gp41 structure revealed that it is a six-stranded helical bundle with an interior coiled-coil trimer of three parallel helices. This feature is very similar to that of the influenza protein HA2 subunit (Chan, Fass et al. 1997; Weissenhorn, Dessen et al. 1997). Activation of the gp120 spike provokes extension of the coiled-coil structure and exposure of gp41 that then fuses with the target cell membrane. After the virus entry into the target cells, the viral RNA genome is reverse transcribed by RT to proviral DNA. RT uses a host cellular transfer RNA (tRNA<sup>lys</sup>) as a primer to make negative strand DNA. The tRNA<sup>lys</sup> possesses a sequence
complementary to the 5’ R-U5 region of the viral LTR. A short viral RNA fragment generated by RNase H from the negative RNA strand primes the synthesis of the positive strand of DNA. The double-stranded HIV DNA is maintained in a nucleoprotein complex with the matrix protein, IN, and RT. It is subsequently transported into the host nucleus. The double stranded DNA then integrates into the host chromosome mediated by the viral IN. Unlike other retroviruses that have very little target sequence specificity for integration sites, HIV-1 preferentially integrates into or beside two classes of repeated DNA elements called L1 and Alu elements (Bukrinsky, Sharova et al. 1993).

In the first few days after infection, the virus replicates freely in the regional lymphatics and subsequently passes into the blood. The virus seeds throughout the body and further concentrates in the lymphatic tissues including spleen, gut-associated lymphoid tissue (GALT), and lymph nodes. The first stage after HIV infection is called acute infection. Viremia is usually reduced to low levels by 3 to 4 months due to the activation of the adaptive immune system including both humoral and cell-mediated immune responses. The humoral response is characterized by the appearance of high titers of HIV-specific antibodies, which are found at or soon after the time of the plasma viremia peak (Figure 1.4). The antibodies decrease HIV replication and plasma viremia through: 1) complement-mediated lysis; 2) antibody-dependent cellular cytotoxicity (ADCC); and 3) complement receptor-dependent trapping in the follicular dendritic cell (FDC) network of lymphoid tissue germinal centers. The cell-mediated immune response plays a critical role in the successful control of plasma viremia (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994).
After acute infection, a second phase, called the ‘clinical latency period’, occurs and usually lasts for several years. The word “latency” hides the fact that the immune system and the virus are both replicating at tremendously rapid levels, each attempting to gain the upper hand. It was estimated that a daily production rate of approximately $10^{10}$ HIV-1 particles is required to sustain the steady-state equilibrium during the latency period (Simon and Ho 2003), while the proliferation rate of CD4+ T cells in an infected individual is about 1 to $2 \times 10^9$ cells/day (Ho, Neumann et al. 1995; Mohri, Bonhoeffer et al. 1998). The CD4+ T cells are killed by 1) direct cell killing whereby large amounts of virus bud off from the cell surface; 2) syncytia formation, in which the infected CD4+ T cells fuse with nearby uninfected CD4 cells to form giant multi-nucleate cells; 3) apoptosis, where both infected and uninfected cells are killed possibly through HIV Env and Tat proteins causing cellular dysregulation and miscommunication with CD8+ cells; 4) direct CTL lysis of infected CD4 cells; and 5) Nef induced apoptosis of the non-infected Fas-expressing T cells via the interaction of Fas and FasL.

Eventually, viral replication causes CD4+ cell numbers to progressively decrease to the point where CD4+ T cell help function is compromised, which causes immunodeficiency leading to eventual death by opportunistic infections. The CD4+ T cell count is used as a measure of disease progression with less than 200 CD4+ T cells per ml in blood as a clinical diagnosis of AIDS. The viral load measurement is another important laboratory test used to monitor the health of HIV-infected patients and their responses to antiretroviral drugs. Viral load is defined as the number of copies of viral RNA per ml of plasma measured by Q-PCR or bDNA (i.e., branched DNA) methods (McDermott, Terrin et al. 2005). A high viral load (> 5,000 copies/ml) indicates that infection is progressing towards AIDS.
Interestingly, neutralizing antibodies (NAbs) typically appear at a later time during the transition from the acute phase to the chronic phase. NAbs are defined as any antibodies produced by the host which can effectively block the HIV-1 infection. These NAbs are usually low titer and slow to develop (Moore, Cao et al. 1994; Burton 1997; Parren, Moore et al. 1999). This could be due to mainly two reasons. First, it takes time to develop somatic mutations of Ig genes for antibody affinity maturation. Second, as the disease progresses, neutralization escape mutants arise as a result of constant and high level mutational diversity (Homsy, Meyer et al. 1989; Homsy, Meyer et al. 1990; Wei, Decker et al. 2003). Therefore, the induction of NAbs may be beneficial later after the acute phase of HIV-1 infection to control viral load but appears to have less influence on disease progression during the chronic infection stage due to the frequent appearance of viruses that express neutralization escape phenotypes.

Escape mutations occur mostly in the glycosylation sites and variable loops of env gp120. N-linked glycosylation sites can be shifted by mutation. Blocked and/or altered epitopes, through shifted glycosylation, allow mutated viruses to escape from NAbs (Wei, Decker et al. 2003). Gp120 has five variable loops (V1 - V5), four of which (V1 - V4) are exposed on the viral surface and shield the conserved core of the protein. Escape mutations can usually be selected against the NAbs that bind these surface variable loops.

The GALT is the initial principal site where HIV-1 replicates and most CD4+ T cells are infected and killed (Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004). This phenomena is also true in the SIV infected monkey model (Li, Duan et al. 2005). GALT CD4+ T cells have, on average, 13-fold more HIV-1 viral DNA and 10-fold more HIV-1 RNA than peripheral blood CD4+ T cells during acute and early stages of the clinical latency period of HIV-1 infection.
HIV-1 RNA was found in both “activated” and “resting” mucosal CD4+ T cells in GALT (Mehandru, Poles et al. 2007). More recently, a new receptor integrin $\alpha_4\beta_7$ and putative co-receptor LFA-1 (lymphocyte function associated antigen-1) were found to promote GALT CD4+ T cell infection. Integrin $\alpha_4\beta_7$ is preferentially expressed on gut CD4+ T cells as a homing receptor for CD4 cells. Although integrin $\alpha_4\beta_7$ was initially found to facilitate HIV-1 infection of NK cells which do not have detectable CD4 expression, HIV-1 gp120 could also bind to CD4+ cells through integrin $\alpha_4\beta_7$ in a CD4-dependent pathway. By sequence comparison and alanine scanning mutagenesis, it was demonstrated that integrin $\alpha_4\beta_7$ binds to a conserved short tripeptide Leu-Asp-Val in the gp120 V2 loop. Binding gp120 with integrin $\alpha_4\beta_7$ may allow HIV-1 binding to receptor CD4 and co-receptor, and then enter GALT CD4+ T cells more efficiently. Binding gp120 with $\alpha_4\beta_7$ on CD4+ cells also results in rapid activation of LFA-1, which promotes the formation of a stable junction or synapse between neighboring cells. The synapse increases the efficiency of cell-to-cell spreading of HIV-1 (Arthos, Cicala et al. 2008).

### 1.1.4 Current treatment

Over the last two decades, extensive research has been aimed at developing anti-HIV drugs that interfere with specific aspects of the viral life cycle. More than 30 drugs have been approved by the FDA which target multiple steps in the infection process. These drugs include: 1) entry inhibitors (including fusion inhibitors such as T20); 2) protease inhibitors such as crixivan; 3) reverse transcription inhibitors (including nucleoside inhibitors such as AZT); and 4) integrase inhibitors. More than 10 other candidates are in experimental development (Roberts, Bebenek et al. 1988; Hamer 2004; Pett S 2008). Multiple drug combinations called Highly Active Antiretroviral Therapy (HAART) have greatly reduced virus replication and disease progression in individuals since its introduction in 1996 by David Ho. However, mutational drug resistance,
patient compliance, side effects, and cost are still major problems limiting this therapy (Romano, Venturi et al. 2002; McDermott, Terrin et al. 2005). More importantly, HAART does not prevent the spread of HIV via individuals with undiagnosed HIV infections. Therefore, the search for an effective vaccine has become an important part of the struggle to fight this disease, and may be the only true way to halt the AIDS pandemic. A vaccine can either be prophylactic or therapeutic. A prophylactic vaccine protects uninfected individuals, while a therapeutic vaccine prevents or eases the severity of the problems in infected individuals. My thesis mainly focuses on development of prophylactic HIV-1 vaccines.

1.2 HIV-1 vaccines

1.2.1 Obstacles to developing an effective HIV-1 vaccine

Despite more than two decades of basic and clinical research, there is still no efficacious HIV-1 vaccine. The virus persistently replicates in the infected individual, leading to disease despite the above-mentioned vigorous humoral and cellular immune responses. HIV-1 possesses multiple strategies to evade host immune surveillance. First, HIV-1 rapidly mutates during infection (~ 1 base per genome per round of replication) (Roberts, Bebenek et al. 1988), resulting in “virus swarms” of divergent sequences that replicate within an individual (Wei, Ghosh et al. 1995). These virus swarms provide fertile ground for selection of immune escape mutants. Secondly, HIV-1 integrates into the host genome and can persist indefinitely in a latent proviral DNA form for the lifetime of the infected individual (Finzi, Blankson et al. 1999). The provirus is capable of re-activating and subsequently replicating in individuals at later times when immune pressures are low or absent (e.g. HAART discontinued) (Brooks and Zack 2002). Thirdly, the target for neutralization (gp120) is a weakly immunogenic molecule due to its unique tertiary structure. The crystal structure of gp120 showed that the CD4 receptor-binding site (CD4BS) is recessed and
flanked by variable regions exhibiting considerable glycosylation (Wyatt, Kwong et al. 1998; Wyatt and Sodroski 1998; Kwong, Wyatt et al. 2000). In addition, the chemokine co-receptor binding site also appears to be masked by variable loops (Wyatt, Moore et al. 1995). The trimeric structure of the active gp120 spike complex shields potentially immunogenic epitopes within its core, while monomeric gp120 virion “debris” elicits largely non-neutralizing antibody responses (Burton and Parren 2000). For these reasons, subunit vaccines based on gp120 have not shown protective efficacy in large-scale human trials (Berman 1998; Francis, Gregory et al. 1998; Cohen 2003). Fusion intermediates generated by cross-linking gp120 with CD4 did not elicit high concentrations of broadly cross-reactive neutralizing antibodies to date (Fouts, Godfrey et al. 2002; Varadarajan, Sharma et al. 2005). Lastly, since the fusion intermediates are short lived, the target epitopes are transient in nature and sterically constrained at the cell membrane surface, which limits antibody access. Therefore, the obstacles that vaccine developers face are truly daunting.

While the obstacles confronting antibody-mediated neutralization are profound, the cell mediated response faces similar problems. Humans have at least three leukocyte antigen (HLA) class I alleles (HLA-A, HLA-B, HLA-C), while rhesus monkeys commonly have the corresponding A and B alleles (Mamu-A and Mamu-B). Each allele binds a different repertoire of viral peptides and presents these for subsequent recognition and elimination by the TCR expressed on CTLs. Due to the high mutation rate of HIV-1 and robust production of virus (~ 10^{10} new viral particles/day), every nucleotide within the viral genome could possibly carry at least one new mutation each day for the 9.5 kb genome. Therefore, the tremendous viral sequence variation can result in loss of or reduced recognition of MHC I (major histocompatibility complex class I)
presented viral epitopes by the CTLs. The process of T-cell selection of these MHC I epitope mutants is known as CTL escape.

HIV-1 and SIV natural infection give rise to CTL escape viruses during both acute and chronic phases. For example, David Watkins and colleagues have studied CTL escape epitopes in Env and Nef during the course of SIV disease progression in rhesus macaques (Evans, O'Connor et al. 1999). Macaque A developed CTL responses directed against three different CTL epitopes (Env575-583, Nef136-146, Nef62-70), while macaque C and D developed CTL responses against 5 (Env497-504, Nef165-173, Env575-583, Nef136-146, Nef62-70) and 3 (Env497-504, Nef165-173) epitopes, respectively. From the early stage of the inoculation to the time of animal death, the plasma virus populations showed amino-acid substitutions in all 10 epitopes. The most frequent variants in the plasma virus populations at the time of animal death had decreased MHC class I binding and/or CTL recognition demonstrated by CTL function assays. These observations demonstrated that CTL selection pressure was driving the appearance of escape mutants. Additional elegant evidence came from passage studies between HLA class I-disparate individuals by the same research group (Friedrich, Dodds et al. 2004). Selected CTL escape mutants were lost from a heterogeneous SIV isolate upon passage in animals that lacked the MHC class determinants to recognize the epitopes. It seems that escape from CTL surveillance comes at the cost of viral fitness since the mutant viruses were lost in the competing viral swarm over time. CTL escape has also been documented in other genes such as gag and tat (Phillips, Rowland-Jones et al. 1991; Allen, O'Connor et al. 2000). More importantly, CTL escape viruses are associated with rapid progression to AIDS (Borrow, Lewicki et al. 1997; Price, Goulder et al. 1997; Allen, O'Connor et al. 2000; Evans, Jing et al. 2000). Recent studies using vaccinated rhesus monkeys challenged with a pathogenic hybrid SHIV virus demonstrated that the frequency
of viral mutations within CTL epitopes correlated with the level of viral replication (Barouch, Kunstman et al. 2002). A single nucleotide mutation within a Gag CTL epitope in one animal with undetectable plasma viral RNA resulted in a loss of viral control by CTLs, a burst of viral replication, clinical disease progression, and eventual death from AIDS-related syndromes. These data indicate that viral escape from CTL recognition is another primary obstacle for effective immune control of HIV-1 and design of successful vaccination strategies.

1.2.2 Nonhuman primate model for HIV-1 vaccine development

Despite these difficulties, the nonhuman primate (NHP) model has provided invaluable information for pre-clinical evaluation of HIV-1 vaccine strategies over the last two decades. Actually, the first strain of SIV was discovered in captive rhesus macaques (SIVmac) only two years after the reported identification of HIV-1 as the cause of AIDS (Daniel, Letvin et al. 1985; Letvin, Daniel et al. 1985). There are several reasons why the SIV/macaque model is useful for vaccine development. First, infection with SIV induces AIDS-like illness over a period of several months to years (Gardner 2003). Secondly, HIV-1 and SIV share similar genetic organization and biological properties that allow for generation of novel chimeric viruses called SHIVs. These SHIVs were generated by inserting the tat, rev, vpu, and env genes of HIV into the genome of SIVmac239 (Shibata, Kawamura et al. 1991; Lu, Brosio et al. 1996; Reimann, Li et al. 1996; Batten, De Rose et al. 2006). Inoculation of macaques with SHIVs results in persistent infection, rapid CD4+ T cell depletion, followed by an AIDS-like illness. These viruses can be used to challenge rhesus monkeys and directly test Env-based HIV vaccines. Thirdly, infection with SIV or SHIV also leads to similar disease outcomes: declining peripheral CD4+ T cell counts, opportunistic infections, wasting, and persistent infection. Fourthly, both SIV and HIV-1 viruses
use similar receptor and co-receptors such as CD4 and CCR5. Lastly, adaptive immunity is elicited and evaded in a similar manner.

1.2.3 Early HIV vaccine development

The first HIV-1 vaccine was tested on nine chimpanzees in 1987 (Hu, Fultz et al. 1987). The animals were immunized with a recombinant vaccinia virus encoding the Env protein of one HIV primary isolate and followed by challenge with either a high or a low dose of the same virus. Though the immunization stimulated HIV-specific antibodies and T-cell responses, virus was found in lymphocytes of all immunized chimpanzees. Vaccinia virus was chosen as the gene transfer vector due to the ability to incorporate additional genetic material because of its large genome size (encodes for about 250 genes). After its use for the eradication of smallpox, this virus was widely studied as a tool for delivering genes into biological systems. Since chimpanzees infected with HIV did not show progress towards AIDS, little was learned from this initial challenge study. In addition to being ineffective, concerns surrounding the use of this vector system in immunosuppressed individuals were raised following the death of an HIV infected military recruit caused by a vaccinia virus encephalitis (Redfield, Wright et al. 1987).

Soon after, vaccine strategy using an inactivated virus was evaluated. In 1989, Murphy-Corb and colleagues vaccinated 9 rhesus monkeys with formalin-inactivated whole SIV particles with the adjuvants alum, Syntex with threonyl muramyl dipeptide (MDP), or MDP alone. Immunized monkeys developed anti-SIV antibodies. Eight out of nine animals were protected against a challenge dose of 10 MID$_{50}$ (1 MID$_{50}$ is defined as the minimal infective dose of a pathogen that produces infections in 50% of the population exposed to that number of pathogens). Due to the fact that inactive vaccine virus and the challenge virus were genetically identical and the
challenge dose (10 MID₅₀) was much lower than in a similar challenge study (200 - 1000 MID₅₀) (Desrosiers, Wyand et al. 1989), enthusiasm was limited. In addition, it may be dangerous to use inactive whole HIV-1 virus on humans. More examples of HIV-1 vaccine development in the past will be addressed in detail in the following sections.

1.2.4 Live Attenuated vaccines

Although as detailed above, the challenges to developing an efficacious vaccine are significant, success has been documented in SIV/SHIV monkey challenge models using live attenuated vaccines. In SIV or HIV, as mentioned earlier, Nef is not required for replication in vitro, but is important for viral replication and pathogenesis in vivo. Nef deleted mutants can effectively protect monkeys against SIV challenge without causing disease (Daniel, Kirchhoff et al. 1992; Wyand, Manson et al. 1996; Stahl-Hennig, Steinman et al. 2002). Desrosiers and colleagues used a live nef deleted SIV strain to infect six rhesus monkeys. All six animals remained healthy for more than 3 years after infection with the mutated virus. Four of the six animals were challenged with 10 MID₅₀ of wild-type SIV (either SIVmac239 or SIVmac251) two years after the initial inoculation with the attenuated virus. All the vaccinated animals were protected, while twelve control animals infected with wild-type SIV died (Daniel, Kirchhoff et al. 1992). Not unexpectedly, persons infected with Nef-deleted HIV-1 strains develop AIDS symptoms much more slowly than those infected with regular HIV-1 strains (Dyer, Ogg et al. 1999; Gorry, McPhee et al. 2007). These results suggested that effective humoral and/or cell-mediated immunity can be mounted and the virus can be cleared from the circulation. Therefore, an attenuated HIV-1 with a nef deletion could potentially be a valuable vaccine candidate for pharmaceutical intervention in AIDS progression. It is noted, however, that the potential for reversion to pathogenicity makes this strategy less attractive for human application (Stahl-
Hennig, Dittmer et al. 1996; Learmont, Geczy et al. 1999). First, the vaccine could revert to the wild type and rapidly cause disease due to overlap of the nef gene with the 3’ LTR and env region. Secondly, the attenuated vaccine itself could cause AIDS after a long time due to restoration of the nef, at least in some individuals (see below); finally, long-term persistence of the attenuated vaccine could cause autoimmune disease or malignancy.

While some of the concerns are theoretical, nine long-term survivors infected with an attenuated, nef-deleted HIV strain in the Sydney Blood Bank Cohort (SBBC) were observed (Dyer, Ogg et al. 1999; Gorry, McPhee et al. 2007). The SBBC cohort consisted of a blood donor and eight transfusion recipients infected by the same attenuated delta-nef HIV strain. Two of them died of non-AIDS-related causes, one died of AIDS-related syndrome due to an immunosuppressive treatment for an autoimmune disease, and the other six, including the donor, have been studied for more than 20 years. All six have remained asymptomatic. While 3 have undetectable viral loads, the other 3 including the donor have detectable viral loads and decreased CD4+ T cell counts. Since the nef ORF overlaps with the 3’ LTR and env region in the HIV genome and covers more than 50% of the nef gene, recombination and mutation could possibly restore a nef ORF leading to a more virulent HIV/SIV strain. A longitudinal analysis of nef and LTR sequence changes in five of these infected persons, including the donor, revealed that a gradual loss of nef sequence occurred, although it varied in magnitude between subjects. These viruses containing nef deletions appeared to be evolving in a convergent fashion toward virus strains with highly deleted, minimal nef/LTR sequences which may be absolutely essential for HIV replication (Churchill, Rhodes et al. 2006). Therefore, nef deletion is beneficial for infected individuals.
1.2.5 T cell vaccines

In the past several years, success in controlling virus replication has been achieved with a variety of genetic vaccines through priming of potent T-cell responses in animal models. These vaccines have elicited strong and often durable antigen-specific T cells, and have been effective at reducing viral burden after challenge in the macaque model (Barouch, Santra et al. 2000; Amara, Villinger et al. 2001). For example, Letvin and colleagues vaccinated 12 rhesus monkeys with a DNA vaccine expressing SIVmac239 Gag and HIV-1 89.6p Env by intramuscular (IM) injections. Eight vaccinated monkeys also had the treatment augmented by the administration of either purified fusion protein IL-2-Ig(Fc) (four monkeys) or a plasmid encoding IL-1-Ig(Fc) (four monkeys). The augmented vaccinated monkeys developed significant levels of antigen specific CTLs that could be detected by tetramer staining after immunization. After the monkeys were challenged by the intravenous route with 100 MID₅₀ (the 50% monkey infectious dose) of cell-free SHIV-89.6, all eight vaccinated monkeys benefited with potent immune responses. They monkeys had preserved CD4+ T cell counts, low to undetectable viral loads, and no evidence of clinical disease. In contrast, six of the eight control monkeys injected with control DNA developed weak immune responses, rapid and profound CD4+ T cell loss, high viral loads, and rapid disease progression after challenge. Only two of the four monkeys vaccinated with the DNA vaccine alone achieved some benefit.

Robinson and colleagues used heterologous prime/boost regimens to control the highly pathogenic SHIV-89.6P that was challenged intra-rectally (Amara, Villinger et al. 2001). The plasmid DNA used for the priming immunization expressed SIV Gag, Pol, Vif, Vpx, Vpr, and HIV-1 Env, Tat, Rev in a single transcript. The booster used a recombinant modified vaccinia Ankara which expressed SIV Gag, Pol, and HIV-1 Env under the control of vaccinia virus
early/late promoters. Rose and colleagues used live attenuated vesicular stomatitis virus (VSV) encoding HIV-1 env and gag to vaccinate seven rhesus monkeys (Rose, Marx et al. 2001). Animals were intravenously challenged with 30 MID\textsubscript{50} SHIV-89.6P. All seven vaccinees were infected with the challenge virus but have remained healthy up to 14 months with low or undetectable viral loads, while the eight control monkeys had a severe loss of CD4\(^+\) T cells and high viral loads. Seven of them progressed to an AIDS-like syndrome with an average time of 148 days. These promising animal data led to a recent clinical phase II trial (Merck trial, more detail in later sections) using a trivalent Ad5 based vaccine expressing clade B gag, pol, and nef genes. A CTL immune response was expected to block or blunt new HIV infections. Unfortunately, it has failed to either prevent HIV-1 infection or suppress viral load in subsequently infected individuals.

Using a recombinant virus as a genetic vaccine to stimulate CTL response is another strategy, which has received intense interest in recent years. Generally, recombinant viral vectors are constructed by removing part or all gene sequences encoding viral proteins and have spaces to accommodate foreign genes of interest. The resulting vectors are usually replication defective and have been proven safe tested in human trials (Clayton, Battinelli et al. 2004; de Bruyn, Rossini et al. 2004; Cebere, Dorrell et al. 2006). Recombinant human adenovirus type 5 (rAd5) is the most used recombinant virus vector. Adenovirus (Ad) is a non-enveloped double-stranded DNA virus, which has an icosahedral capsid with twelve vertices and seven surface proteins. Its genome encodes about thirty proteins. Ad infection causes respiratory infections and induces common colds, intestinal and eye infections in humans. Currently, about 51 human adenovirus serotypes have been discovered, of which adenovirus serotype 2 and 5 have been the most widely studied and used in gene transfer applications (Wu, Dmitriev et al. 2002; Yan, Kitzes et al. 2003;
Ad is particularly attractive as a vaccine vector because: 1) large amounts of highly purified recombinant virus can be easily produced; 2) the genome of adenovirus is episomal, so it provides a safe environment to use as a gene transfer vector; 3) the virus can infect a large variety of dividing and quiescent cells including antigen presenting cells; and 4) the virus can accommodate large transgene inserts up to 7.5 kb. First generation rAd5 vectors contain deletions of the E1 and E3 early genes that are complemented in trans using cell lines (293 or PerC6). This yields a replication incompetent vector, with no viral dissemination. The culmination of rAd5 genetic vaccine development was the Merck Phase II STEP trial that used a trivalent rAd5 vaccine expressing HIV-1 gag, pol, and nef genes. Results from this large multi-center, randomized, double blind, placebo-controlled phase II clinical trial indicated that the vaccine failed to protect Ad5 sero-negative individuals from HIV-1 infection (Clayton, Battinelli et al. 2004). After three years of testing with 3,400 volunteers, two conclusions were drawn: 1) the vaccine did not prevent infection; and 2) the vaccine did not reduce viral load in the plasma of those who were infected. Furthermore, the vaccine may even have facilitated HIV-1 infection in the vaccinees with pre-existing immunity to Ad. It has been postulated by some researchers that the vaccine stimulated an immune response that yielded greater numbers of activated CD4+ cells to serve as infection targets (Cohen 2006; Cohen 2007). In addition, some researchers suggested that omission of the env gene in Merck’s vaccine limited the breadth of immunity and might have also contributed to the trial’s failure.

An interesting question raised by the failure of the STEP trial was the validity of correlates of protection against HIV-1 infection. The vaccine elicited high numbers of antigen specific T-cells based on an IFN-γ ELISpot assay, indicating that cell-mediated immunity was elicited. Significantly, the ELISpot and related intracellular cytokine staining assays have been used
widely in HIV-1 vaccine development, including the STEP trial (Casimiro, Wang et al. 2005; De Rose, Taylor et al. 2005). However, these methods only measure the ability of CD8+ T cells to secrete INF-γ in response to high peptide concentrations. Neither assays directly measure the ability of CTLs to suppress the replication of HIV-1 in CD4+ T cells.

Heterologous prime-boost immunization strategies have also been widely used in the field of HIV-1 vaccine development. In most cases, a single dose of a vaccine induces weak and short-term immune responses that provide ineffective protection. Repeated administration (boosting) of the same antigen can increase the immune response by causing expansion of memory T-cells generated during the initial prime. A traditional prime/boost method consists of administering the same antigen in two or more doses, which is called a homologous prime-boost. A heterologous prime-boost uses two different vectors to dose the same antigen, which could potentially elicit immune responses with greater potency and breadth than the homologous prime/boost method. In preclinical models, this strategy has been used in developing vaccines against malaria (Schneider, Gilbert et al. 1998), cancer (Irvine, Chamberlain et al. 1997), and tuberculosis (McShane, Brookes et al. 2001). In 2003, Ertl and colleagues constructed a chimpanzee adenovirus vaccine vector (AdC6) encoding a truncated form of HIV-1 gag. This construct could induce high frequencies of Gag-specific CTLs in mice, even in those pre-exposed to human Ad5 virus. More importantly, more than a 10-fold higher frequency of Gag-specific CTLs was achieved by using the construct and human Ad5 vector with the prime-boost strategy (primed with AdC6-gag, boosted with human Ad5-gag) (Pinto, Fitzgerald et al. 2003). A recent study in the SIV/monkey model demonstrated that prime-boost with plasmid DNA (encoding either gag/pol, env, or gag/pol and env) and rAd5 (encoding gag/pol and env) could provide a reduction in viral load and increase survival time after the animals were challenged with SIVmac251 through an intravenous
route (Letvin, Mascola et al. 2006). The vaccination regimens elicited high levels of SIV-specific CTLs with better responses detected in the monkeys which received the DNA prime/rAd5 boost than those that received only rAd5 immunization.

Beside rAd, recombinant adeno-associated virus (rAAV) is another viral vector that has recently been used in prime/boost strategies (Johnson, Schnepp et al. 2005). Compared to rAd, the rAAV vector is simpler, much more versatile, and safer. There are few distinct serotypes based on antibody reactivity to the virus capsid in which serotype 2 has been the most intensively used. More details on rAAV will be discussed later in Section 1.4. Dr. Clark and colleagues vaccinated a total of 21 rhesus monkeys using DNA or rAAV2 prime and rAAV2 boost in an IV challenge model (Johnson, Schnepp et al. 2005). The DNA plasmids encoded either: 1) SIV subgenomic fragments of the *gag* and protease; 2) reverse transcriptase and integrase; or 3) the *rev* and *env*. The rAAV2 viral vectors encoded the same genes. The study utilized either a DNA or rAAV2 prime with a mixture of three plasmids or viral vectors. Both groups received the same rAAV2 *rev-env* boost. The monkeys were challenged intravenously with a live, virulent SIV post vaccination. The monkeys with the best control of SIV replication post-challenge were in the DNA prime and rAAV2 boost group despite a weakly immunogenic DNA prime. Both the viral load at peak and set point from this experimental group were 100-fold lower than that from the control group. Although NAbs were produced with the above strategy, the protection from challenge was attributed to CTLs since the sera from vaccinated animals did not significantly neutralize the challenge virus stock on the challenge day. Based on these pre-clinical studies, several HIV vaccine clinical trials are currently underway to evaluate the safety and immunogenicity (phase II), and efficacy (phase III) of prime-boost immunization strategies even in light of STEP trial results (Table 1.1).
1.2.6 NAb vaccines

Usually a HIV-1 infected individual mounts a powerful antibody response to the virus. However, the process of antibody maturation is slow and high-titer neutralizing antibodies often take years to elicit. In fact, all human viral vaccines in use today that provide sterilizing immunity against infections do so through elicitation of neutralizing antibody responses. However, no immunogens have been found or made so far which can elicit humoral immune responses to prevent HIV-1 infection even for a single subtype.

Clearly, proteins that encode portions of the HIV pathogen could theoretically be used as subunit vaccines as has been successfully demonstrated by the human papillomavirus (HPV) vaccine targeting HPV (Bailey and Cymet 2006). This approach has also been successfully used in preparation of a licensed preventive HBV (Hepatitis B virus) vaccine in yeast (Valenzuela, Medina et al. 1982) and a candidate Herpesvirus type II vaccine (United States Patent 3897549). Using this approach, the first two HIV protein vaccines (Vax003 and Vax004) were Env-based subunit vaccines conducted by VaxGen, Inc. from 1998 to 2003 (Phase III clinical trials). Vax004 used a fusion protein from the two antigens in the same subtype B, while Vax003 used a fusion protein from subtype B and E. The first trial Vax004 was conducted in North America and Europe since subtype B is prevalent in those regions, while the Vax003 trial was conducted in Bangkok, Thailand where subtypes B and E are prevalent (Berman 1998; Flynn, Forthal et al. 2005). Despite producing neutralizing and CD4-blocking antibodies in all of the vaccinees, the vaccines were ineffective in preventing HIV infection or in modifying post-infection markers of disease progression. One reasonable explanation is that the NAbs elicited were not able to neutralize genetically diverse primary HIV viruses. Another factor was the monomeric forms of recombinant gp120 used in both vaccines. Since the natural envelope glycoprotein spike exists as...
a trimer on the viral surface, the monomer may not retain critical conserved structures that induce broadly NAbs against primary HIV-1 isolates. Conformational accurate Env-trimer antigens have been constructed (gp120-gp41), but the weak association between gp120 subunits (Grundner, Mirzabekov et al. 2002; Sanders, Vesanel et al. 2002) has resulted in poor immunogenicity (Yang, Wyatt et al. 2001; Grundner, Li et al. 2005; Bower, Li et al. 2006).

However, this is not to imply that neutralizing antibodies are not critical to controlling HIV-1 infection. Dennis Burton and colleagues as well as Katinger and colleagues have identified several potent NAbs from seropositive, non-progressing patients, using phage display technology (Burton, Barbas et al. 1991; Zwick, Labrijn et al. 2001; Moulard, Phogat et al. 2002; Scanlan, Pantophlet et al. 2003). These NAbs (X5, IgG1b12, 2G12, 2F5, and 4E10) target the following epitopes on the Env trimer: IgG1b12 recognizes a discontinuous epitope that overlaps the CD4BS; 2G12 recognizes a conserved carbohydrate structure; 2F5 and 4E10 recognize linear epitopes on gp41 near the fusion domain; and X5 binds to the CD4i epitope (Figure 1.5) (Burton, Pyati et al. 1994; Zwick, Wang et al. 2001; Moulard, Phogat et al. 2002; Burton, Desrosiers et al. 2004; Zwick, Jensen et al. 2005). Phage display is a selection technique in which a library of peptides or antibodies are expressed on the surface of a phage virion, while the genetic material encoding each variant is inserted into the phage genome. Such a library can be screened rapidly by panning against a target antigen based on binding affinity. Accordingly, Burton and colleagues constructed several human Fab (fragment of antigen binding) libraries made from the cDNA of HIV infected bone marrow derived mRNA and screened these library for Fabs that bind to HIV env gp120 or the gp120-CD4-CCR5 complex (Burton, Barbas et al. 1991; Zwick, Wang et al. 2001; Moulard, Phogat et al. 2002).
Once these critical antibodies were identified, a series of passive immunization studies were undertaken to address the importance of pre-existing NAb in helping to control SIV/HIV infection. Several studies have clearly shown that these pre-existing NAbs do play a critical role in limiting viral dissemination during the early stage of infection, and result in complete protection (sterilizing immunity), or reduction in viral burden with an increase in survival time (Mascola, Louder et al. 1997; Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999; Mascola, Stiegler et al. 2000; Mascola, Lewis et al. 2003). These experimental data not only proved that shared neutralization-sensitive viral domains do exist, but also demonstrated that these domains can be effectively used to block virus infection. NAbs act either by binding to the mature gp120 Env trimer to block initial receptor engagement or by binding to gp41 after virion attachment to inhibit the fusion process. For X5, it appears that during infection, the virus transiently exposes the co-receptor binding site possibly in a sterically limited manner, with the consequence that smaller forms such as the single-chain variable-fragment antibody X5 (scFv X5) and Fab versions show better neutralization than the full length IgG molecule. Virus neutralization appears to require essentially all of the functional trimers to be occupied by at least one antibody molecule (Yang, Kurteva et al. 2005), which implies that the concentration of NAbs is a critical factor. The passive immunization studies argue that plasma NAb levels between 50 - 200 μg/ml at the time of virus challenge may be sufficient to provide complete protection in many vaccinees, and result in reduced viremia and increased CD4+ levels in those that do become infected. All attempts to elicit high titer NAbs with the same specificities as these NAbs such as 2F5 by pre-defined epitope vaccination have been unsuccessful (Joyce, Hurni et al. 2002; Burton, Desrosiers et al. 2004). However, if we could supply an individual with a high enough concentration of one or more of these existing broadly NAbs in the blood constantly, he/she may be completely protected or have a reduced viral burden. It is not practical to administer these NAbs by passive
immunization to millions of people over their lifetime. Thus, the challenge for the field is how to provide broadly cross-reactive NAbs at sufficient concentrations to prevent HIV-1 viral infection over a long period of time.

1.3 Antibodies

Since my approach has focused on the generation and delivery of single-chain variable-fragment (scFv) based NAbs against HIV-1/SIV, the particular characteristics of antibodies that are relevant to HIV-1/SIV NAb gene based vaccine design are detailed below.

1.3.1 Antibodies structure, function, and production

Antibody molecules, also known as immunoglobulins (abbreviated as Ig), are generally composed of two light chains (kappa or lambda) and two heavy chains (alpha, delta, epsilon, gamma, or mu). Five distinct classes of immunoglobulin (IgG, IgA, IgM, IgD, and IgE) are found in higher animals, which are defined by the heavy chain constant region. All immunoglobulins have a similar structure despite their different size, charge, amino acid (AA) composition and carbohydrate content. IgA and IgM exist as monomeric and/or multimeric forms depending on a joining (J) chain via the tail region of their heavy chain. IgM is the largest (950 kD) immunoglobulin since it forms a pentamer after secretion. Membrane bound IgM serves as the antigen receptor of naive B cells and is involved in B cell maturation and activation of the classical pathway of complement. IgA exists in monomeric or dimeric forms, and functions mostly in mucosal and neonatal passive immunity. IgD is a major component of the surface membrane of many B cells and functions as an antigen receptor. IgE is mainly expressed on the membrane surface of basophils and mast cells, and is involved in IgE-mediated allergic reactions. In humans, IgG is the most abundant (75% of the plasma antibody in adults) and long-lived
antibody among the five classes, with a half-life of up to 21 days in serum, while the other 4 have half-lives on the order of 2 to 6 days in serum. IgG exerts its physiologic functions via binding to extra-cellular microbes, microbial toxins, and virus particles. Many of these functions are mediated by the Fc (fragment, crystallizable) domain and different Ig heavy chain isotypes have distinct effector functions. A summary of antibody functions and properties is listed in Table 1.2.

As shown in Figure 1.6 and 1.7, IgG is made up of two identical light chains and two identical heavy chains linked by a variable number of disulfide bonds. The light chains fold into two domains (V_L and C_L), while the heavy chains fold into four domains (V_H, CH1, CH2, CH3). The N-terminal domains of V_H and V_L recognize the antigen, while the other domains compose the constant regions. In the heavy chain, a flexible spacer domain called the “hinge” is located between CH1 and CH2. The stem of the antibody includes the hinge, CH2, and CH3 and is referred to as the Fc domain. The length and flexibility of the hinge region varies among the four IgG subclasses and affects their susceptibilities to proteolytic enzymes, such as papain, plasmin, trypsin, and pepsin. Functional sub-units of the antibody molecule were revealed by the use of enzymatic digestion. The protease papain cleaves the Fc domain yielding a Fab antigen binding molecule that has the complete light chain and V_H–CH1 fragment. Pepsin cleavage also removes the Fc domain but the two Fab arms are still connected by a disulfide linkage yielding a F(ab’)_2 fragment. Recently, developments in the field of molecular biology have allowed the generation of even smaller moieties that retain antigen binding properties and these are called scFv antibody (Bera, Kennedy et al. 1998; McHugh, Hu et al. 2002; Afanasieva, Wittmer et al. 2003). A scFv consists of the V_L and V_H linked by a short peptide linker. Because of its small size (~ 30 kD), the scFv molecule has rapid bio-distribution and tissue penetration properties compared to a complete antibody, leading to its use as an in vivo targeting/visualization molecule (Yokota, Milenic et al. 2002).
We have focused on scFv-IgG1 and IgG2 fusion molecules in the following Chapters due to the extended natural half-life of these proteins and the ability of high-titer circulating IgG to passively traffic to the surface of the mucosal epithelium from the serum (transudation) as a way to blunt or prevent experimental SHIV/SIV infection.

Upon exposure to a new antigen, naïve B cells are activated with additional signals from helper T cells. The B cells then further differentiate into either plasma B cells or memory B cells depending on T cell-derived cytokines. Plasma B cells are professional antibody secretion cells which can secrete Ig in the absence of an antigen. The lifespan of plasma cells are relatively short and last only several days to weeks. Memory B cells, on the other hand, tend to live longer and can therefore respond quickly upon a second exposure to an antigen. B cells may also go through an intermediate differentiation step in the lymphoid germinal center, where somatic hypermutation of Ig genes in variable domains and further isotype switching occurs. It was initially demonstrated in hybridoma cultures that with time and successive exposure of antigen, somatic mutations dramatically increase the affinity of an antibody for its cognate antigen via germ-line mutation (Berek, Apel et al. 1989). Isotype switching, also called class switching, is the process in which activated IgM and IgD expressing B cells switch heavy chain isotype to IgG, IgA, or IgE under the influence of cytokines by switch recombination. Class switch recombination is a process in which the VDJ gene segment of an antibody (Fab and hinge region) recombines with a different constant region and the intervening DNA is deleted.
1.3.2 IgG subclasses, receptors, and their effector functions

1.3.2.1 IgG subclasses

In humans, IgG is further divided into 4 subclasses (IgG1, IgG2, IgG3, IgG4) (Table 1.2). IgG1 and IgG2 are the two most abundant subclasses in humans among the four sub-classes (Abbas AK 2000). IgG1 and IgG2 make up 89% of the total IgG antibody population. Except IgG3, the other subclasses have a serum half-life ~ 21 days. As described earlier, IgG is the major effector molecule of the humoral immune response. All recombinant antibodies developed for therapeutic applications are from the IgG class. Therefore I will mainly discuss details related to IgG subclass.

In IgG subclasses, the two main functions mediated by Fc are ADCC and complement-dependent cytotoxicity (CDC). Quantitatively, the relative serum concentrations of the human IgG subclasses are in the order of IgG1, IgG2, and IgG3 from high to low. The serum concentrations of IgG3 and IgG4 are similar (Shakib and Stanworth 1980). The four subclasses show more than 95% homology in protein sequences in the Fc regions. However, their hinge regions are different in amino acid composition and length, which determines the flexibility of the molecule (Burton, Gregory et al. 1986). As Figure 1.7 shows, the two identical antigen-binding Fab fragments of each IgG subclass molecule and its single Fc fragment are quite mobile. Specifically, the hinge region of IgG1 is composed of 15 AA residues (216 to 231). The Fab fragments of IgG1 can rotate freely around their axes of symmetry and move within a sphere centered at the first of the two heavy chain disulfide (S-S) bridges (Brekke, Michaelsen et al. 1995). IgG2 has a shorter hinge region than IgG1 (12 AA long) and has four inter-heavy chain S-S bridges. The short hinge contains a rigid poly-proline double helix, which is further stabilized by the four S-S bridges. Therefore, the flexibility of an IgG2 molecule is limited (Abramov, Arkhangelskaya et al. 1983).
IgG3 has the longest hinge region among the four subclasses (62 AAs including 21 prolines and 11 cysteines), which is almost three times longer than the hinge of IgG1. The proline and cysteine rich fragment forms an inflexible poly-proline double helix (Saluk and Clem 1971; Michaelsen, Naess et al. 1993). Because of the extended hinge region, the Fab fragments of IgG3 are relatively far away from the Fc fragment, which gives the molecule the maximum flexibility. The length of the hinge region of IgG4 is the same as that of IgG2 with only two S-S bridges, and its flexibility is between that of IgG1 and IgG2. The structural differences among the four IgG subclasses are directly related to their susceptibilities to proteolytic enzyme digestions. IgG3 is very susceptible to cleavage by these enzymes while IgG2, IgG1, IgG4 are relatively resistant, which also explain their relatively longer half-lives (Table 1.2).

1.3.2.2 IgG Fc receptors

IgG Fc receptors (FcγRs) are almost all integral membrane glycoproteins. Cloning and sequence analysis have shown that the FcγRs are structurally related and contain conserved Ig-like domains. The extracellular regions of all the FcγRs are homologous and the genes encoding FcγRs are almost all located in the same region of chromosome 1. FcγR1 has been identified on monocytes and macrophages in humans and mice. IFN-γ can up-regulate the receptor expression. Fc engagement with the FcγR on macrophages, monocytes, myeloid cells, and dendritic cells is the initial step in effector function signal transduction. For human IgG subclasses, there are three distinct FcγRs: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). Each of these receptors has multiple isoforms. FcγRI is a high-affinity receptor which is expressed on most effector cells and has a high affinity for monomeric human IgG1 and IgG3. It has a 10-fold lower affinity for IgG4 and does not bind IgG2. FcγRII is expressed on most types of blood leukocytes as well as on Langerhans cells and platelets. It is the only receptor that binds IgG2. Three genes encode three
different forms of this receptor (FcγRIIA, FcγRIIB, and FcγRIIC). FcγRIIA and FcγRIIB have a low affinity to all subclasses of monomeric IgG, while FcγRIIC’s binding property has not been determined yet. FcγRIIB is expressed exclusively by lymphocytes, especially B cells. FcγRIIB is the only inhibitory receptor expressed in humans. B cell activation is shut down when polyvalent IgG1 or IgG3 engages FcγRIIB (Hulett and Hogarth 1994).

Compared to FcγRI and FcγRII, FcγRIII has intermediate affinity for IgG. FcγRIII has two isoforms, FcγRIIIa and FcγRIIIb. Both of the isoforms bind IgG1 and IgG3. FcγRIIIa is expressed on macrophages, monocytes, natural killer cells, and subsets of T cells. It has the unique ability to interact with lectins from bacteria. The neonatal Fc receptor (FcRn) is a heterodimer composed of β2-microglobulin and a transmembrane α-chain. This receptor not only permits passive transfer of maternal IgG to the fetus but also regulates IgG homeostasis by re-circulating IgG in the systemic circulation. Binding of IgG to FcRn targets the protein to endosomes (rather than lysosomes) that are then recycled to the cell surface. pH dependent binding of IgG with FcRn is a hallmark of the FcRn interaction, with a high-affinity binding at acidic pH (pH < 6.6) and weak or no binding at neutral pH. Based on this mechanism, several mutants of IgG were constructed which showed longer half-lives due to the high affinity binding to FcRn (Hinton, Xiong et al. 2006). A summary of these receptor properties is listed in Table 1.3.

1.3.2.3 Effector functions

IgG subclasses are divided according to their distinct Fc sequences. As a consequence, each subclass shows unique effector functions in complement activation, opsonisation, and induction of phagocytosis. During complement activation, a cascade of reactions occurs involving factors C1 to C9 to form a membrane attack complex (MAC) on a target cell. The target cell is then
killed by lysis. The classical pathway of CDC involves the binding of C1q to the sites on the CH2 domain of IgG. This binding starts when an antigen-antibody complex forms. Human IgG3 has the strongest binding to C1q followed by IgG1, IgG2, and then IgG4 (Flanagan and Rabbitts 1982). Subclass IgG4 actually does not activate complement while subclass IgG3 is the most effective activator. The flexibility of the hinge region may determine the accessibility of the complement-binding site to C1q. The density and accessibility of antigenic determinants on the microbe surface also contribute to the actual complement activation by IgG subclasses.

Another mechanism used to eliminate microbes is ADCC. When IgG binds to an antigen on target cells, the Fc region of that IgG can subsequently bind to cell surface FcγRs expressed on immune effector cells such as macrophages and natural killer cells, leading to microbe phagocytosis by macrophages or lysis of the targeted cell by natural killer cells. Opsonisation is the process by which antibodies coat a microbe to facilitate phagocytic cell uptake. ADCC is efficiently stimulated by IgG1 and IgG3. Activation of the complement component C3 can also result in opsonisation of microbes by complement components C3b, iC3b, and C4b, which leads to greater internalization by phagocytic cells. Phagocytes then engulf the microbe particles after opsonisation and form an internal phagosome. The phagosome is usually delivered to the lysosome for microbe degradation. For genetic vaccine design, usage of different subclasses and mutational substitutions might be used to avoid or enhance ADCC or CDC effector functions.

1.3.2.4 Glycosylation and stability

All IgG molecules are modified by glycosylation in the constant regions at conserved positions of the heavy chains. Each isotype possesses a distinct array of N-linked carbohydrate structures that affect antibody assembly, secretion and functional activities (Gala and Morrison 2002). IgG has a
conserved single N-linked glycosylation at Asn297 in the CH2 domain. The Asn297 glycan is buried between the CH2 domains, forming extensive contacts with amino acid residues within the CH2 and non-covalently paired CH3 domains. The sugar residues are attached through Asn297 as a biantennary core structure (Wright and Morrison 1997; Jefferis, Lund et al. 1998). Oligosaccharide attachments are also found in the Fab region at non-conserved glycosylation sites (Sox and Hood 1970; Spiegelberg, Abel et al. 1970). The frequencies and locations depend on the presence of Asn-X-Ser sequences in the hypervariable regions in V_H and V_L. Crystal structure studies of human IgG show that the Asn297 oligosaccharide has a distinct conformation (Sauer-Eriksson, Kleywegt et al. 1995; Corper, Sohi et al. 1997). In sharp contrast, several other glycoproteins bearing a single N-linked oligosaccharide (e.g. chicken avidin and ovotransferrin) in which the oligosaccharide is mobile and does not confer thermal stability (Wang, Eufemi et al. 1996). This unusual conformation forms multiple non-covalent interactions with the protein, such that the immunoglobulin and oligosaccharide each has a reciprocal influence on the conformation of the other. Therefore, alterations in antibody glycosylation patterns affect antibody thermal stability and antigenicity due to conformational changes in the protein (Parekh, Dwek et al. 1985; Mimura, Church et al. 2000). Depending on the isotype, aglycosylated IgG typically has a shorter in vivo half-life due to an open conformation in the protein molecule, leading to increased solvent accessibility and proteolytic attack (Tao and Morrison 1989). Therefore, the production of correctly glycosolated antibodies in cells other than B cells is an important consideration for our study, in which we use muscle as an ectopic production site.

1.3.3 Antibody immunogenicity

Antibody from one individual could be immunogenic to the others in the same species. Unique antigenic determinants on individual antibody molecules are termed idiotypes. Antibodies against
idiotypes are called anti-idiotypic antibodies. Usually, an anti-idiotypic antibody is against the hypervariable loop regions (HVR) of the Fab fragment, and may also include some of the framework residues near the heavy and light chain’s HVRs. Since we propose to produce a single antibody at modest levels, the expressed antibody could elicit anti-idiotypic antibodies. These anti-idiotypic antibodies in turn could stimulate anti-anti-idiotypic antibodies and so forth generating a network of anti-antibodies. It is theorized that a subset of these antibodies may carry an “internal image” of the original antigen and therefore the anti-anti-idiotypic antibody may have a similar binding specificity of the original antibody. Through variable domain and anti-variable domain interaction, various antibody generations either stimulate or suppress the production of one another (Figure 1.8). This idiotypic recognition and elicitation cascade has been termed the “Network Hypothesis” by Nobel laureate Niels Jerne in 1974 (Jerne 1974) (Nobel Prize winner in medicine in 1984). To date, experimental data indicates that anti-idiotypic antibodies and anti-anti-idiotypic antibodies indeed exist (Cazenave 1977; Comacchio, Bradley et al. 1996). For example, anti-RNase antibodies from one rabbit were used to raise anti-idiotypic antibodies in a second rabbit, which were then used to raise anti-anti-idiotypic in a third animal that was structurally similar to the initial anti-RNase antibody (Cazenave 1977). In another study, a mAb against human albumin was used to immunize rabbits. All rabbits developed both anti-idiotypic and anti-anti-idiotypic antibodies concomitantly. The anti-anti-idiotypic antibody was similar to the mAb in terms of affinity and specificity for a proteolytic fragment of human albumin (Comacchio, Bradley et al. 1996).

The possibility of eliciting anti-idiotypic antibody responses in other animal models such as mice or monkeys also exists with therapeutic antibodies (Reth, Imanishi-Kari et al. 1979; Schneider, Glaser et al. 1993; Tosi, Valota et al. 1996; Kunert, Weik et al. 2002). It is an intriguing
possibility that a potent anti-HIV NAb response composed of a few immunodominant antibodies could stimulate anti-idiotypic and anti-anti-idiotypic antibodies. This anti-idiotypic antibody response could potentiate our approach based on the network hypothesis. In this scenario, the anti-idiotypic antibodies would mimic epitopes on the envelope glycoprotein that are of low inherent immunogenicity. These in turn stimulate anti-anti-idiotypic antibodies that would be predicted to neutralize HIV-1. In fact, a report has documented the generation of a murine anti-idiotypic mAb (Ab2/3H6) to the human HIV-1 neutralizing antibody 2F5 (Kunert, Weik et al. 2002). Ab2/3H6 Fab fragments were able to induce a neutralizing immune response in mice. Importantly, these mouse sera exhibited *in vitro* neutralization potency against a HIV-1 laboratory strain. Therefore, the impact (both positive and negative) that anti-idiotypic antibody responses may play is worthy of consideration in our antibody expression studies.

An ideal HIV vaccine is an immunogen that elicits antibodies which broadly neutralize field strains of HIV-1 virus. Unfortunately, as mentioned earlier, no such antigen has been found or designed. Consequently, we have explored the advantage of using the few broadly NAbs which have been already identified. An infusion or injection of antibodies every few weeks is neither practical nor cost effective as a large-scale prophylactic vaccine. We need a novel delivery system.

### 1.4 Recombinant AAV vectors and antibody gene delivery

Since our laboratory has been interested in viral-mediated gene transfer, it occurred to us that it might be possible to transfer potent NAb genes into a host to provide some levels of protection from HIV-1 infection. Ideally, transduced cells would produce NAbs constantly and the host would be effectively protected for a long time (Figure 1.10). To accomplish antibody gene
transfer, we have chosen to focus on rAAV vectors. rAAV vectors can mediate robust, long-term *in vivo* gene transfer in organs composed primarily of non-dividing cells such as muscle, liver, brain, and eye (During and Leone 1995; Carter and Samulski 2000; Li, Miller et al. 2008; Wu, Sun et al. 2008).

### 1.4.1 AAV biology and its usage as a gene transfer vector

Wild-type adeno-associated virus (wtAAV) is a small (25nm), non-enveloped, non-pathogenic, and replication-defective parvovirus. There are at least 8 distinct serotypes based on antibody reactivity to the virus capsid. Serotype 2 (AAV2) has been the most intensively studied. The AAV2 genome is composed of 4.7 kb single-stranded DNA and encodes two genes *rep* and *cap*. The *rep* and *cap* genes are flanked by highly structured inverted terminal repeats (ITR). The two 145 bp ITRs contain signals for genome replication, packaging, site-specific integration, and circularization. rAAV can be made by replacing the viral internal *rep* and *cap* genes with an eukaryotic expression cassette. Rep and Cap are supplied in *trans* by helper plasmids or viruses (such as wtAAV) (Rabinowitz, Rolling et al. 2002). Therefore, rAAV vectors contain no native viral genes (Figure 1.9). Virus particles can be produced by transient transfection in 293T or HeLa cells. rAAV can also be produced from a producer cell line (Liu, Clark et al. 1999; Liu, Wagner et al. 2003). The particle is very stable and has a buoyant density at 1.41 g/ml. Therefore, it can be easily purified using density gradients and column chromatography (Clark, Liu et al. 1999; Zolotukhin, Byrne et al. 1999).

The AAV life cycle starts with an infection by using receptors and/or co-receptors, which vary for the different serotypes. Upon introduction into the host nucleus, the single stranded AAV genome is converted into double stranded DNA episomes. AAV propagates in cells in two stages. With
the co-infection of helper virus such as adenovirus, or herpes simplex virus, AAV produces progeny virus by a lytic cycle. In this cycle, AAV undergoes genome replication, viral gene expression, and virion production. Adenovirus supplies helper functions for AAV growth from a subset of its gene products that include E1a, E1b, E2a, E4, and VA RNA. Herpesvirus can also provide help by producing DNA polymerase, helicase, and the early functions necessary for virus transcription. In cell culture, AAV infects cells and is maintained as a provirus at a site termed AAVS1 on chromosome 19 (q13.4). The latent AAV provirus can be rescued from the chromosome and re-enter the lytic cycle if cells are super infected with a helper virus.

AAV was first considered as a gene transfer vector based on the ability of the wild type virus to integrate in cultured cells (Samulski, Zhu et al. 1991). However, recent data indicate that integration is inefficient \textit{in vivo}. Extensive studies of monkey tissues in our laboratory demonstrated that more than 99.5% AAV genomes persist as a non-integrated extra-chromosomal form (Schnepp, Clark et al. 2003). It was shown that these extra-chromosomal DNAs persisted over time as transcriptionally active monomeric and concatameric episomes \textit{in vivo} using a sensitive PCR assay (Schnepp, Jensen et al. 2005). Rep gene is neither included in the virus vector genome nor the protein packaged inside the virus that is required for the AAV-S1 integration. Further studies demonstrated that the ability of rAAV to persist as episomes depends on host cellular enzymes such as DNA-PK which is used to convert the linear viral DNA into stable circular and concatemeric forms (Song, Laipis et al. 2001; Choi, McCarty et al. 2006). Similar results were obtained in mouse liver injected with rAAV2 encoding human factor IX (hF.IX) (Nakai, Yant et al. 2001) and mouse muscle injected with rAAV2 encoding eGFP (Duan, Sharma et al. 1998). Not surprisingly, Dr. Flotte and colleagues demonstrated that DNA-PK inhibits AAV DNA integration in liver tissue using knockout mice (Song, Laipis et al. 2001).
their studies, they injected rAAV2 encoding human alpha 1-antitrypsin into the portal vein of either C57BL/6 (DNA-PKcs<sup>+/−</sup>) or severe combined immunodeficient (DNA-PKcs<sup>−/−</sup>) mice. After partial hepatectomy to stimulate liver regeneration, increased DNA genome copy numbers and higher transgene expression were found in the severe combined DNA-PKcs<sup>−/−</sup> mice. These results also demonstrated that the non-integrated episomal DNA was lost in dividing cells. Therefore, non-dividing cells such as myofibers are good target for vaccine applications using rAAV mediated gene transfer.

Another attractive property of this vector system is that it appears to be safe. AAV is not associated with any disease in humans, although 70 - 90% of the human population is seropositive for AAV. Recombinant AAV vectors do not contain any viral genes and have been safely administered to more than 500 humans with no severe adverse events in over 20 clinical trials (Wagner, Messner et al. 1999; High 2002; High 2003; Carter 2005; Warrington and Herzog 2006).

1.4.1.1 AAV serotypes and their discovery

AAV was initially observed in the mid-1960s as 25-nm contaminants in several Ad preparations. It was found later that the contaminants were viral particles and not Ad degradation products (Atchison, Casto et al. 1965; Hoggan, Blacklow et al. 1966). From then on, infectious AAV isolates have been obtained from primates, rodents, canines, birds, and even reptiles. There are currently at least 8 distinct primate serotypes (AAV1 - 9) based on antibody reactivity to the virus capsid. AAV2, AAV3, AAV9, and an AAV2/3 hybrid have been isolated from humans. Since the capsids of AAV1 and AAV6 are 99% identical and AAV6 capsids can be neutralized by anti-AAV1 serum, they are not considered to be distinct serotypes (Rutledge, Halbert et al. 1998).
AAV 1/6, AAV4, and AAV8 appear to circulate mainly in NHPs. AAV7 has been isolated from both humans and NHPs. Because of high sequence conservation between AAV capsids, cross-reacting antibodies are frequently observed in primate and human populations (Grimm and Kay 2003; Halbert, Miller et al. 2006). However, NAbs to the human serotypes are more common in the human population than NAbs to the NHP serotypes, which makes AAV1, 6, and 8 vectors favorable for use in humans (Xiao, Chirmule et al. 1999). The prevalence of AAV2 and AAV3 neutralizing antibodies were 70 to 80%, respectively, while AAV1 serum NAbs are present only in about 20% of adults (Rapoza and Atchinson 1967).

Since AAV2 is the prototypic serotype, most vector development work has focused on it. Actually, AAV2 was the first rAAV cloned (Samulski, Berns et al. 1982). AAV3 was found in SV15 preparations and shares 82% sequence homology with AAV2. AAV4 is most likely a monkey specific AAV since it was only isolated from African green monkeys infected with simian adenovirus SV15. Humans do not appear to have antibodies against AAV4. AAV5, identified originally from a human clinical sample, was thought to be sexually transmitted in association with the herpes simplex virus. This serotype is the most divergent human AAV serotype since it has only about 60% homology to AAV2. AAV6 was isolated as a contaminant of an SV5 stock and was believed to be either a variant of AAV1 or a natural recombinant between AAV1 and AAV2 (Xiao, Chirmule et al. 1999). AAV7 and AAV8 were both isolated recently from rhesus monkey tissues by PCR using highly conserved primers in cap gene (Gao, Alvira et al. 2002). Using similar PCR techniques, AAV9 was subsequently found in human tissue by the same research group (Gao, Vandenberghhe et al. 2004). Interestingly, antibodies against AAV9 do not cross react with the first 6 serotypes, which demonstrated that AAV9 is serologically distinct from other known serotypes (Table 1.4). Recently, our laboratory found that
9 out of 175 human pediatric tissues including freshly collected tonsils and frozen samples from spleen, lung, muscle, liver, and heart were found to contain AAV DNA sequences. Complete capsid gene sequences recovered from eight of these AAV-positive tissues were AAV2-like (98% amino acid identity), while a single spleen isolate was intermediate between serotypes 2 and 3 (Chen, Jensen et al. 2005). More recently, our laboratory isolated 25 new wild-type AAV clones from 28 rhesus monkey tissue samples (Jenson, et al, paper submitted). Most of the DNA sequences have high homology with the known serotypes AAV4, 7, and 8 that circulate in the species. Four unique capsid clones rh.73, rh.74, rh.75, rh.76 were identified, though they may not be serologically distinct from the currently identified serotypes 1 to 9. New serotypes are expected to be found to enrich our choices for gene therapy application with more studies on AAV.

1.4.1.2 AAV molecular structure

Knowledge of the genome structure of AAV has mostly come from studies of AAV2 (Harter, Robenkranz et al. 1969). The genome encodes two genes - rep and cap. The rep gene encodes 4 proteins Rep78, Rep68, Rep52, and Rep40, which are involved in virus DNA replication and regulation of viral transcription and translation. The two larger Rep proteins Rep78 and Rep68 are produced from transcripts initiated at the P5 promoter, while the smaller proteins Rep52 and Rep40 are produced from transcripts using the P19 promoter. Rep78 and rep68 regulate AAV gene expression in the presence or absence of helper virus and are required for DNA replication. Rep52 and Rep40 are required for the accumulation of single-stranded viral DNA for packaging within AAV capsids. The cap gene encodes 3 overlapping proteins VP1 (732 AAs, 90 kD), VP2 (596 AAs, 72 kD), and VP3 (530 AAs, 60 kD), which form the virus capsid. The three-capsid proteins are expressed from one overlapping ORF with different start locations driven by the
AAV p40 promoter. At the protein sequence level, the three-capsid proteins differ only in their N-terminal sequence length. VP3 is the major capsid protein efficiently translated from an AUG start code and represents about 90% of the total virion protein. X-ray crystallography studies revealed that each capsid is made up of 60 subunits of VP1, VP2, and VP3 in proportions of about 3:3:54 (Xie, Bu et al. 2002).

Since the virus encapsidates a single-stranded genome, in order to express the inserted gene the host cell has to synthesize the complementary strand. Several studies have shown that second strand synthesis can be a rate-limiting process for efficient transgene expression (Ferrari, Samulski et al. 1996; Fisher, Gao et al. 1996; Natkunarajah, Trittibach et al. 2008). To by-pass the requirement for this host cell function, investigators are pursuing the potential advantages of self-complementary (SC) AAV vector in which a DNA hairpin is packaged. The hairpin DNA can rapidly form a double stranded circular form inside the nucleus. These vectors are generated by mutating one of the ITR regions at the terminal resolution site (McCarty, Monahan et al. 2001). Further studies demonstrated that SC vectors mediate more rapid and higher transgene expression than the regular single stranded rAAV vectors in vivo (Fu, Muenzer et al. 2003; McCarty, Fu et al. 2003; Gao, Lu et al. 2006). However, one disadvantage of the SC vector system is that it can only accommodate a transgene less than 2.2 kb including the promoter and polyadenylation sequences. For scFv antibodies (e.g., scFv X5) which are usually less than 1 kb, SC vectors are an ideal choice.

1.4.1.3 rAAV in vivo gene transfer

As defined earlier, rAAV can be made by replacing the viral internal rep and cap genes with an eukaryotic expression cassette. Rep and Cap are supplied in trans by helper plasmids or viruses.
Rep is from AAV2. Cap candidates are from these 8 serotypes and up to 100 sequence variants. Advantages and the tissue tropisms of each are under intense investigation. AAV1, AAV6, and AAV8 appear promising as viral vehicles for gene delivery to muscle cells (Wang, Zhu et al. 2005). rAAV1 has been shown to be one of the most effective vectors for muscle specific gene transfer. In Table 1.4, I summarize the optimal target tissues and the known receptors for the principle serotypes. The ability to cross blood vessel barriers makes AAV6 and AAV8 a very attractive gene therapy vector for systemic delivery to muscle, heart, liver, spleen and pancreas (Nakai, Fuess et al. 2005; Cheng, Wolfe et al. 2007). More recently, hybrid vectors between different serotypes such as an AAV1-AAV2 hybrid have also emerged. Dr. Weidong Xiao’s research group demonstrated that AAV-221-IV, a hybrid vector generated by the replacement of some amino acids in AAV2 VP1 by the corresponding amino acids in AAV1, increased muscle directed gene delivery efficiency while still preserving the heparin binding property of AAV2 (Hauck, Xu et al. 2006). Since the virus capsid is assembled by 60 monomers of the VP1, VP2, and VP3, Dr. Rabinowitz and colleagues mixed different AAV capsid helper plasmids to generate “cross-dressed” AAV virions (Rabinowitz, Bowles et al. 2004). Some of these transencapsidated virions produced interesting phenotypes for custom-designed AAV vectors. Peptide insertion into discrete capsid locations is also a powerful approach to gain new properties for the old vector (Girod, Ried et al. 1999; Shi, Arnold et al. 2001).

1.4.2 rAAV immunogenicity

1.4.2.1 Host immune responses to vector itself

rAAV was initially viewed as a relatively non-immunogenic vector based on limited immune responses in inbred rodent models, a factor which usually influenced vector transduction in a positive manner. Accordingly, a relatively restrained innate immune response to the capsid
proteins has been documented (Fisher, Jooss et al. 1997; Zaiss, Liu et al. 2002). Intravenous injection of rAAV vectors in mice induced low-level transient production of pro-inflammatory cytokine and leukocyte infiltration into the liver. However, the soluble cytokines and cellular infiltration did not last longer than six hours. By contrast, more immunogenic viruses, such as Ad, induce strong innate responses lasting 24 hours or longer (Schnell, Zhang et al. 2001; Zhang, Chirmule et al. 2001). Failure to actively induce innate signaling pathways may contribute to reducing adaptive immune responses to the foreign particle. Since the vector is extremely simple and encodes only the transgene, adaptive immune responses target either the virus capsid or the transgene itself. The virus particle is known to stimulate robust anti-capsid humoral immunity in all animals. In fact, 18 - 67% of the human population has antibodies capable of significantly neutralizing rAAV1 - 5. The pre-existing immunity could potentially be a problem for using these serotypes directly in gene therapy in human clinical trials.

Capsid immunogenicity of AAV could be due to the repeating structure of the viral particle. For example, in AAV2, more than 10 highly repetitive linear and complex conformational epitopes were identified in the viral capsid which can induce NAbs (Moskalenko, Chen et al. 2000; Wobus, Hugle-Dorr et al. 2000). Anti-AAV NAbs are very potent in blocking vector re-administration with the same serotype in mouse models (Riviere, Danos et al. 2006). Other studies were carried out using pooled human intravenous immunoglobulin IgG (IVIG) in a mouse model. Transgene expression after IM administration was reduced 5-fold for rAAV1 and 10-fold for rAAV8 in IVIG-pretreated mice compared to a novel caprine serotype (rAAV-Go.1) (Arbetman, Lochrie et al. 2005). Transgene expression after intravenous (IV) administration was reduced 7.1-fold for rAAV-Go.1, 3.8-fold for rAAV8, and completely extinguished for rAAV2 in mice pretreated with even less IVIG. Novel capsids from nonprimate AAVs may be another
approach to avoid neutralization by human pre-existing antibodies. Chemical modification of the surface of the AAV vector such as PEG conjugation is another way to lower the immunogenicity of the vector and prevent NAb elicitation (Lee, Maheshri et al. 2005). More recently, protein evolution techniques using synthetic shuffled capsid libraries have led to novel rAAV vectors with reduced immunogenicity (Maheshri, Koerber et al. 2006). As detailed in Section 1.4.1.3, mixing capsids and insertion peptides are also good strategies to create new serotypes to avoid immune responses.

The cell-mediated immune response to the virus vector is poorly understood and has been largely ignored until recently. A clinical trial conducted by Dr. Katherine High’s group demonstrated that AAV capsid induced cell-mediated immunity might have cleared transduced hepatocytes in a patient receiving an AAV2 vector expressing human factor IX (hF.IX) (Manno, Pierce et al. 2006). This phenomena was not identified in their pre-clinical studies with a dog model for hemophilia B (Mingozzi and High 2007). Subsequent studies in healthy donors revealed that humans have a population of AAV capsid specific memory CTLs, which are probably induced by previous wild-type AAV exposure. These cells appear to be expanded upon administration of the rAAV vector and might lead to the clearance of the rAAV transduced liver cells. Possible solutions which could avoid this response include: 1) using alternative serotypes or novel synthetic serotypes; 2) using immuno-suppressive drugs such as rapamycin and cyclophosphamide; 3) pre-screening for naïve subjects; and 4) reducing the viral antigen load by improving the quality and potency of the virus. One example of improving potency would be optimization of the gene expression cassette (e.g., use of a strong promoter) allowing for a lower viral dose to achieve the same expression levels. Improving viral production and purification processes to lower the empty viral particles in final viral stock preparations may also reduce the
viral antigen load. While anti-capsid immunity is clearly a concern, it remains to be determined if transduced cells are always efficient targets for T-cell mediated clearance. For instance, Ertl and colleagues demonstrated that pre-existing AAV capsid-specific CTLs were unable to eliminate AAV-transduced hepatocytes in a mouse model (Li, Murphy et al. 2007). These capsid-specific CTLs had lytic activities on AAV capsid epitope-loaded target cells in vitro. It is most likely that mouse hepatocytes had a limited capability to process and/or present AAV capsid proteins, which failed to activate capsid-specific CTLs. These results are in contrast to the outcome of the recent gene therapy clinical trial conducted by Dr. Katherine High’s group as mentioned above. It is possible that immune mechanisms other than CD8+ T cells such as NK cell killing and/or ADCC effect may result in loss of AAV-transduced cells in the patient’s liver. The finding may also reflect differences in antigen processing and presentation in mouse and human hepatocytes.

### 1.4.2.2 Host immune responses to transgene

Immune responses to the transgene vary according to the particular protein expressed. The response also relates to: 1) transgene expression level; 2) target tissues transduced; 3) promoter used to drive the gene expression; 4) route and site of administration and expression; 5) host genetic background; and 6) self vs. non-self antigen. Several well-studied examples are discussed below to illustrate that multiple variables can impact the observed immunological outcomes.

Immune responses against factor IX (F.IX) have been well studied in several animal models. Both IM injected AAV2 encoding hF.IX in mice (Herzog, Hagstrom et al. 1997) and canine F.IX in dogs (Herzog, Mount et al. 2001) elicited humoral responses to F.IX that prevented F.IX from reaching detectable levels in the circulation. In the mouse model, NAbs against human F.IX were produced in immune-competent C57BL/6 mice but not in immuno-deficient Rag-1 mice, in
which a therapeutic level of human F.IX in plasma was reached. In one dog model, canine F.IX was expressed in hemophiliac dogs carrying a null mutation of F.IX, which had an early stop mutation and unstable mRNA. Therefore, for these dogs, canine F.IX was still a neo-antigen. In another dog model with a mis-sense mutation, intramuscular injection demonstrated that increasing the dose per injection site correlated with an increase in inhibitory antibody response (NAb to F.IX) (Herzog, Fields et al. 2002). Serotype 1 IM injections in both mice and dogs also resulted in an inhibitor (antibody) formation (Arruda, Schuettrumpf et al. 2004). Transient immune suppression with cyclophosphamide at the time of vector administration inhibited anti-canine F.IX antibody formation in one treated animal.

Based on these pre-clinical studies on rAAV2/CMV.hF.IX injection in mouse and dog muscle mentioned above, the first clinical trial using the rAAV2/CMV.hF.IX vectors was carried out in subjects with mis-sense mutations in the F.IX gene (Kay, Manno et al. 2000). Three patients were dosed from $2 \times 10^{11}$ to $1.8 \times 10^{12}$ vg/kg into 10 - 12 sites in the vastus lateralis of either one or both legs. F.IX expression was detectable for over three years based on immunofluorescence staining of biopsied injected muscle. No neutralizing antibodies against hF.IX were produced and pre-existing high-titer antibodies against AAV2 did not prevent gene transfer or expression (Manno, Chew et al. 2003). Although circulating hF.IX levels did not persistently rise above 1% (defined as a efficacy level for all the patients dosed, the actual levels in the low dose patients were higher than that predicted by mouse and dog studies. These observations may suggest that the vector is more efficient in humans than in mice or dogs. Since AAV2 was originally isolated from humans, the processes of vector binding and entry may be more efficient in human cells than in those of other species (Summerford and Samulski 1998). Another reason could be the CMV promoter-
enhancer is more efficient in humans since the virus may have evolved to work most efficiently in the setting of human transcription factors.

In contrast with muscle delivery for a null mutation of F.IX, liver-directed canine F.IX gene transfer in F.IX null dogs induced a tolerogenic environment for the transgene and no NAbs were produced in several animals (Mount, Herzog et al. 2002). This phenomenon also holds in mice injected with vectors containing human F.IX at similar doses with different promoters (Mingozzi, Liu et al. 2003). It is most likely that the liver microenvironment promotes tolerance rather than immunity to hF.IX (Knolle and Gerken 2000). First, the liver is the natural site of biosynthesis of clotting factors, so unlike in muscle, the necessary post-translational modifications in the protein encoded by the transgene may be made correctly; secondly, since liver is a highly vascularized organ, newly synthesized proteins can be easily and efficiently transported to the systemic circulation; and thirdly, as mentioned above, the expression of foreign proteins in transduced hepatocytes is associated with induction of tolerance rather than immunity. Therefore, for some foreign proteins, the liver might be a better choice to express transgene than muscle tissue.

As predicted by these studies in mouse and dog models, the liver may be a better target for F.IX. Accordingly, the clinical trial study of liver delivery of a rAAV encoding hF.IX under the control of a liver-specific promoter achieved higher transgene expression for at least two months than that in a clinical trial using muscle delivery. Three dose cohorts (8x10^{10}, 4.0x10^{11}, 2x10^{12} vg/kg) were infused through the hepatic artery in seven subjects. The four subjects who received the first two doses showed no evidence of vector-related toxicity or efficacy. The fifth subject, subject E who received the dose of 2x10^{12} vg/kg, was expected to reach efficacy levels. In fact, this subject showed therapeutic levels of hF.IX expression in the range of 10 - 12% of circulating F.IX for 3
weeks. However, the hF.IX level began to fall at the beginning of week 4 and gradually dropped to baseline (< 1%) at week 10 after the vector infusion. Accompanying with the decline of hF.IX, the subject experienced a transient asymptomatic elevation of liver transaminases. Non-inhibitory antibodies against hF.IX were found in this subject. Cell-mediated immunity (CMI) against the AAV2 capsid was demonstrated. This CMI might be responsible for the decline of hF.IX and the liver transient transaminitis as detailed in the Section 1.4.1.4.

Then, why were CTL responses not observed against F.IX in the clinical trial using muscle injection? First, the virus targeted different tissues. The liver is actually an immune organ which contains mainly parenchymal cells (i.e., hepatocytes). One of the nonparenchymal cells, the liver sinusoid endothelial cell (SEC), are located on the walls of the hepatic sinusoid. This cell may function as an antigen-presenting cell (APC) which usually induces antigen-specific T cell tolerance (Kmiec 2001). Liver macrophages also modulate immune responses via antigen presentation, suppression of T-cell activation, and participate in the development of oral tolerance. However, in the presence of capsid-specific CD8+ memory T cells, these cells may reactivate a cellular immune response (Mingozzi, Maus et al. 2007). On the other hand, muscle tissues contain mainly specialized fiber cells, which are not involved in any immune response. Secondly, the virus infected different cells. In muscle injection, the majority of the viral vector usually stays localized around the injection site. Therefore, myofibres are likely the major target cells to be transduced. In the human clinical trial targeting liver, the vector was infused through the hepatic artery. The vectors entered the liver and transduced hepatocytes mainly through the receptor heparan sulfate proteoglycan (Summerford and Samulski 1998). However, this procedure cannot prevent the virus vectors from infusing into blood and infecting APCs in blood circulation through the same heparan sulfate proteoglycan receptor or other mechanisms.
Therefore, not only a liver specific promoter is needed to drive the transgene expression to avoid immune response against the transgene, but also a liver specific vector or procedure for viral transduction to avoid reactivating capsid-specific CD8+ T cells is necessary.

Even for a self-protein, the expression level of the transgene could be problematic in that misfolded over expressed proteins may induce an immune response. In two separate studies, different rAAV serotypes encoding the self-protein rhesus erythropoietin (Epo) were administered to rhesus monkeys by the IM route (Chenuaud, Larcher et al. 2004; Gao, Lebherz et al. 2004). Expression of the Epo protein induced a strong NAb response. In the first study, rAAV1 at two doses 2.4x10^{10} and 6.7x10^{11} vg/kg, rAAV2 at two doses 1.7x10^{11} and 6.7x10^{11} vg/kg, and rAAV5 at a dose of 8.5x10^{10} vg/kg encoding rapamycin or doxycycline regulated rhesus Epo gene were used to inject 9 monkeys (Chenuaud, Larcher et al. 2004). After 3 to 4 induction cycles, most animals produced humoral and CTL responses against Epo resulting in the loss of Epo production and absence of circulating endogenous Epo. Post-translational modification in the muscle tissue due to the over-expression of the Epo protein was suggested as the reason for the immune responses (Arruda, Hagstrom et al. 2001). Likewise, in the study conducted by James Wilson and colleagues, rAAV1, 5, and 8 encoding rhesus Epo driven by the CMV promoter at a dose of 1x10^{13} vg/kg induced humoral and cell mediated immune responses resulting in severe anemia in all 8 injected monkeys. Interestingly, two monkeys injected with rAAV7 at the same dose did not develop anti-Epo immune responses. The exact mechanism responsible for this difference in the AAV7 injected animals remains unclear. This study illustrates that ectopic sites of transgene expression should be closely monitored for retention of native protein folding and glycosylation to minimize the potential for an immune response to improperly processed transgene products.
Another well-studied transgene is *Escherichia coli* β-galactosidase (LacZ gene) that has been extensively studied in animal models following rAAV or rAd transduction. This is a good example of a non-self antigen. IM injection of rAAV2 encoding lacZ (rAAVlacZ) failed to induce transgene clearance in an immuno-competent inbred mouse model (Fisher, Jooss et al. 1997), while the same experiment performed with rAd and naked DNA vectors induced strong T-cell responses that targeted cell muscle fibers for destruction (Wolff, Malone et al. 1990; Jooss, Yang et al. 1998). One explanation was that rAAV failed to enter APCs or rAAV entered APCs but in the form of a non-productive transduction event (Ferrari, Samulski et al. 1996). While antigen-specific T-cell responses appear to not be effective, the foreign transgene elicits antibodies in BALB/c mice administrated with rAAV2.lacZ (Lo, Qu et al. 1999). It was further found that transduction of immature dendritic cells (DCs) is a key event to elicit CD40L-dependent T-cell immunity against the transgene expressing cells (Zhang, Chirmule et al. 2000). When outbred dogs were given intramuscular injections (Yuasa, Yoshimura et al. 2007) of rAAV2 encoding β-galactosidase, only low transient expression of β-galactosidase was observed. Strong humoral and cellular immune responses were found against the transgene product. These studies underscore the importance of the host haplotype, environment, and the transgene itself in the initiation of immune responses against the transgene product.

Additionally, the promoter may influence where and how much of the transgene is expressed. Therefore, regulated or tissue specific promoters, which are not active in APCs, may facilitate escape from immune recognition by avoiding direct antigen presentation on the transduced APC. For similar reasons, lowering the vector dose may limit expression of the transgene below a threshold for initiation of an immune response. Moreover, the target organ itself may affect the immunological outcome. For example, the liver may supply a microenvironment to promote
immune tolerance via induction of suppressive T-cells termed T-regs (CD4+ CD25+ Foxp3+) (Suvas, Kumaraguru et al. 2003; Dobrzynski, Fitzgerald et al. 2006). Consistent with this hypothesis of immune tolerance in the liver is a recent demonstration that adoptive transfer of antigen-specific T-reg cells at the time of gene transfer promotes transgene persistence (Gross, Leboeuf et al. 2003). More recently, using knockout mice, Bartlett, Muruve, and colleagues demonstrated that the complement system also plays an important role in the host immune response to AAV2 (Zaiss, Cotter et al. 2008). In summary, host immune responses have the potential to tremendously impact the success of human clinical gene therapy trials. Immune modulation and vector design are two areas of intense investigation to address possible concerns with pre-existing AAV immunity in the target patient population.

1.5 Statement of hypothesis

The studies presented in this dissertation focus on developing a novel, prophylactic vaccine against HIV-1/SIV by rAAV mediated gene delivery that encode pre-selected, potent, and broadly neutralizing antibodies. Upon systemic secretion of NAbs from transduced skeletal muscle cells by rAAV into the blood circulation, humoral immunity should be present to prevent or significantly blunt subsequent HIV-1/SIV infection. The project is translational in concept, attempting to express these protective neutralizing antibody molecules at therapeutic levels in a relevant non-human primate challenge model, setting the stage for eventual human clinical trial testing.
Table 1.1: HIV vaccines currently testing in Phase II and III clinical trials using prime and boost strategies

<table>
<thead>
<tr>
<th>Official codes (Phase)</th>
<th>Vaccine vector (prime/boost)</th>
<th>Immunogen (clade)</th>
<th>Sponsor and manufacturer</th>
<th>Eliciting T cell and/or NAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV 144(III)</td>
<td>vCP1521/AIDSVA X B/E</td>
<td>env (B, E)</td>
<td>NIAID, Thailand MOPH, VaxGen, Sanofi</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>IAVI A002(II)</td>
<td>AAV (two doses)</td>
<td>tgAAC09 (C)</td>
<td>IAVI, Targeted Genetics</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>HVTN 204 (II)</td>
<td>DNA plasmid/rAd</td>
<td>gag, pol, nef (B), Env (A, B, C), pol (B)</td>
<td>NIAID, Vical, GenVec</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>ANRS VAC 18 (II)</td>
<td>LIPO-5 (three doses)</td>
<td>5 lipopeptides from gag, pol, nef (B)</td>
<td>ANRS, Sanofi Pasteur</td>
<td>T cell only</td>
</tr>
<tr>
<td>RV172 (I/II)</td>
<td>DNA plasmid/rAd5</td>
<td>gag, pol, nef (B), env (A, B, C)</td>
<td>USMHRP, NIAID</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>NCHECR-AE1 (I/II)</td>
<td>DNA plasmid/fowlpox</td>
<td>gag, pol, tat (A, E), rev, env (A, E)</td>
<td>The Univ. Of South whales</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>HIVIS 03 (I/II)</td>
<td>DNA plasmid/MVA</td>
<td>env (A, B, C), gag (B), RT (B), rev (B), env (E), pol (E)</td>
<td>Karolinsk Inst., SMI, Vecura</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>tgAAC09 (II)</td>
<td>DNA plasmid/rAAV</td>
<td>Gag, pol, ΔRT</td>
<td>Targeted Genetics, IAVI</td>
<td>T cell and NAbs</td>
</tr>
<tr>
<td>Class</td>
<td>Subclass</td>
<td>Plasma level (mg/ml)</td>
<td>Molecular secretory form</td>
<td>Serum Half-life (days)</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>----------------------</td>
<td>--------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>IgM</td>
<td>None</td>
<td>1.5</td>
<td>Pentamer</td>
<td>5</td>
</tr>
<tr>
<td>IgG</td>
<td>IgG1</td>
<td>9</td>
<td>Monomer</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>IgG2</td>
<td>3</td>
<td>Monomer</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>IgG3</td>
<td>1</td>
<td>Monomer</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>IgG4</td>
<td>0.5</td>
<td>Monomer</td>
<td>21</td>
</tr>
<tr>
<td>IgA</td>
<td>IgA1</td>
<td>3</td>
<td>Monomer, Dimmer, Trimer</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IgA2</td>
<td>0.5</td>
<td>Monomer, Dimmer, Trimer</td>
<td>6</td>
</tr>
<tr>
<td>IgD</td>
<td>None</td>
<td>Trace</td>
<td>no</td>
<td>2.8</td>
</tr>
<tr>
<td>IgE</td>
<td>None</td>
<td>0.05</td>
<td>Monomer</td>
<td>2</td>
</tr>
</tbody>
</table>
# Table 1.3: Human IgG receptors and their functions

<table>
<thead>
<tr>
<th>Name</th>
<th>Distribution</th>
<th>Affinity</th>
<th>Specificity</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFcγR1</td>
<td>Monocytes, Macrophages, Neutrophils, Eosinophils,</td>
<td>High</td>
<td>3 &gt; 1 &gt; 4</td>
<td>Phagocytosis, ADCC, Respiratory burst, Release inflammatory factors</td>
</tr>
<tr>
<td>(multiple genes, three isoforms)</td>
<td></td>
<td></td>
<td>&gt;&gt; 2</td>
<td>B cell activation, Ab production (For all three type receptors, but different cells have different functions)</td>
</tr>
<tr>
<td>FcγRII</td>
<td>Monocytes, neutrophils, eosinophils, Platelets, B cells</td>
<td></td>
<td>FcγRIIa: High, FcγRIIb: Low</td>
<td>3 &gt; 1 &gt; 2, 4</td>
</tr>
<tr>
<td>(multiple genes: FcγRIIA, FcγRIIB, FcγRIIC, multiple isoforms)</td>
<td></td>
<td></td>
<td></td>
<td>Only FcγRIIB is an inhibitory Receptor</td>
</tr>
<tr>
<td>FcγRIII(2 genes, two isoforms)</td>
<td>Neutrophils, eosinophils, macrophages, NK cells</td>
<td>Low</td>
<td>1 = 3</td>
<td>Passive transfer of maternal IgG to fetus, recycle IgG (FcRn only)</td>
</tr>
<tr>
<td>FcRn</td>
<td>Endothelium Epithelial cells</td>
<td></td>
<td>&gt;&gt; 2 = 4</td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Serotype</th>
<th>Circulates</th>
<th>Receptor</th>
<th>Target tissue/cell</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV1</td>
<td>SV 15 stock NHP</td>
<td>Sialic acid (α-2,3 and α-2,6 N-linked)</td>
<td>Skeletal muscle, retina, pancreas</td>
<td>(Wu, Miller et al. 2006; Cheng, Wolfe et al. 2007)</td>
</tr>
<tr>
<td>AAV2</td>
<td>SV 12 stock human</td>
<td>Heparan sulfate proteoglycan (HSPG) αVβ5 integrin, Growth factor receptors, LamR</td>
<td>Liver, muscle, eye, CNS, alveolar cells, pancreas</td>
<td>(Samulski, Berns et al. 1982; Griffey, Wozniak et al. 2006; Cheng, Wolfe et al. 2007; Li, Miller et al. 2008)</td>
</tr>
<tr>
<td>AAV3</td>
<td>SV 15 stock human</td>
<td>HSPG, Fibroblast growth factor receptor, LamR</td>
<td>Smooth muscle, erythroid, and megacaryoblast cells</td>
<td>(Atchison, Casto et al. 1965; Rutledge, Halbert et al. 1998)</td>
</tr>
<tr>
<td>AAV4</td>
<td>NHP</td>
<td>Sialic acid (α-2,3 O-linked)</td>
<td>CNS/ependyma, astrocyte, airway epithelial cells</td>
<td>(Chiorini, Yang et al. 1997; Kaludov, Brown et al. 2001)</td>
</tr>
<tr>
<td>AAV5</td>
<td>Human</td>
<td>Sialic acid (α-2,3 and α-2,6 N-linked)</td>
<td>CNS, muscle, liver, retina, lung/ respiratory tract, airway epithelial cells</td>
<td>(Kaludov, Brown et al. 2001; Limberis and Wilson 2006)</td>
</tr>
<tr>
<td>AAV6</td>
<td>SV 5 stock NHP</td>
<td>Sialic acid (α-2,3 and α-2,6 N-linked), Heparin</td>
<td>Murine airway epithelial cells, Skeletal muscle, retina, pancreas</td>
<td>(Xiao, Chirmule et al. 1999; Halbert, Allen et al. 2001)</td>
</tr>
<tr>
<td>AAV7</td>
<td>NHP Human</td>
<td>Sialic acid (α-2,3 and α-2,6 N-linked), Heparin</td>
<td>Skeletal muscle</td>
<td>(Gao, Alvira et al. 2002)</td>
</tr>
<tr>
<td>AAV8</td>
<td>NHP</td>
<td>LamR</td>
<td>Liver/hepatocytes, heart, skeletal muscle, pancreas</td>
<td>(Gao, Alvira et al. 2002; Cheng, Wolfe et al. 2007)</td>
</tr>
<tr>
<td>AAV9</td>
<td>Human</td>
<td>LamR</td>
<td>Liver, heart/myocardium, skeletal muscle, pancreas, murine airway epithelial cells</td>
<td>(Limberis and Wilson 2006)</td>
</tr>
</tbody>
</table>
**Figure 1.1: Organization of the HIV-1 virion** (source: [http://www3.niaid.nih.gov/news/newsreleases/2004](http://www3.niaid.nih.gov/news/newsreleases/2004), accessed on March 4, 2008). HIV is about 145 nm in diameter and roughly spherical. Each surface spike is a trimer of three gp120s and three gp41s, both encoded by the *env* gene. Inside of the lipid membrane is the layer of matrix, which is composed of protein p17. Inside of matrix shell is the viral capsid core, which is composed mainly by p24. The HIV genome contains two identical strands of RNA, which are packed within the viral capsid. The reverse transcriptase is the key enzyme for viral replication and sequence diversity.
Figure 1.2: HIV-1 genome organization. (source: http://biology.fullerton.edu/biol302/Browser/moreabout.html, accessed on August 9, 2008). HIV-1 genome encodes for three main regions \textit{gag}, \textit{pol}, and \textit{env} which translate to different core proteins, viral enzymes, and envelope proteins as indicated in the figure. HIV-1 genome also encodes six other accessory genes among \textit{Pol}, \textit{Env}, and 3’-LTR (\textit{tat}, \textit{vif, vpu, vpr, and nef}), some of which are essential for HIV-1 replication and reproduction. The gene \textit{nef} has overlaps with LTR and \textit{env}. 
Figure 1.3: Mechanism of HIV-1 entry into cells (Doms and Moore 2000). The Env trimer is composed of three gp120 and three gp41. Binding of CD4 to the HIV-1 gp120 subunit of Env induces exposure of a conserved region in gp120, which then initiates coreceptor (i.e., CoR, either CCR5 or CXCR4) binding. CD4 binding also appears to trigger exposure of the triple-stranded coiled-coil, and, presumably, exposure of the fusion peptide. Binding to coreceptor brings Env in closer proximity to the target membrane, enabling the fusion peptide to insert in the host cell membrane bilayer. The insertion of the fusion peptide into the cell membrane promotes envelope and cell membrane fusion, so that the HIV genome can then be passed to the target cell. Neutralization antibody such as 2F5 or 4E10 can bind to gp41 and inhibit the fusion process.
Figure 1.4: Immune response to HIV infection, disease progression, and co-receptor usage (Abbas AK, L. A., Pober J.S., 2000). A CTL response to HIV is detectable as early as 2 to 3 weeks after the initial infection and peaks by 9 to 12 weeks. The dramatic reduction in plasma viremia during this early phase of HIV infection is most likely due to the CTL response. The humoral immune response to HIV started at week 6 and peaks at 12 weeks. Unfortunately, most of these antibodies are non-neutralizing antibodies and do not promote virus clearance. When CD4+ T cell counts drop to about 200/mm³, the risk of obtaining accidental infection and other clinical components of AIDS is high. It usually takes years for a HIV positive person to remain asymptomatic. CD4 is the main receptor for virus to get into host cells, while the co-receptor usage can be changed from early CCR5 only to CCR5 or CXCR4. In the AIDS stage, some mutated virus can use both CCR5 and CXCR4 for co-receptors.
Figure 1.5: A model of the HIV-1 envelope spike showing the location of epitopes recognized by several NAbs (Burton, Desrosiers et al. 2004). The three gp120 monomers, constituting the Env trimer, are in shades of blue. The representation of gp41 in green is schematic. It is likely that the 4E10 and 2F5 epitopes are available after gp120 has engaged with the host cellular receptor. IgG1b12 (b12), 4E10, 2F5, 2G12, and X5 (CD4i binding site) are indicated in the figure. Permission to use the figure was granted.
Figure 1.6: Ribbon representation of a crystal structure of IgG (Image source: http://www.antibodyresource.com/intactab.html). The graph is a labeled ribbon drawing of the first crystallized intact mouse antibody IgG2. The red colored ribbon represent light chains, while the yellow and blue ribbons represent two heavy chains. The light chains fold into two domains V L and C L, and the heavy chains fold into four domains (V H, CH1, CH2, and CH3). The Fab is composed of light chain, V H and CH1. The connection between CH1 and CH2 is the hinge region. The stem of the antibody shown at the bottom including the hinge region, CH2, and CH3, is the Fc domain. A conserved single N-linked glycosylation at Asn297 is in the CH2 domain.
Figure 1.7: Schematic structure of the four human IgG subclasses. The four domains of
the heavy chains and the two domains of the light chains are shown (source: http://www.xs4all.nl/~ednieuw/IgGsubclasses/subk123.htm, accessed on Feb. 20, 2008). The hinge region of IgG1 is
composed of 15 AAs, which allows the Fab fragment rotate freely around their axes of symmetry
and move within a sphere centered at the first of the two heavy chain S-S bridges. IgG2 has a
shorter hinge region than IgG1 (12 AA long) but has four inter-heavy chain S-S bridges. The
short hinge contains a rigid poly-proline double helix, which is stabilized by the four S-S bridges.
The flexibility of IgG2 molecule is limited. IgG3 has the longest hinge region among the four
subclasses with 62 AA long including 21 prolines and 11 cycteines. The proline and cysteine rich
fragment forms an inflexible poly-proline double helix. The length of hinge region of IgG4 is the
same as that of IgG2 with only two S-S bridges, and its flexibility is between that of IgG1 and
IgG2.
Figure 1.8: One of the models of network hypothesis. Ab1 (labeled as 1 in the figure) is the antigen recognizing set, Ab2 (labeled as 2) is the anti-idiotypic set, and Ab3 (labeled as 3) is anti-anti-idiotypic clones. Ab1 stimulates B cells to produce Ab2. The V domains of Ab2 function as antigenic epitopes and elicit the synthesis of Ab3. This cascading effect forms a network or series of networks depending upon idio-type-anti-idioype recognition between T and B cells. Through variable and variable domain interaction, the various antibodies generate either stimulation or suppression of the production of one another (Richter 1975).
**Figure 1.9: Constructs of wild-type AAV and recombinant AAV vectors.** Wild-typed AAV contains rep, cap, and two highly structured inverted hairpin repeats (TR), while rep and cap genes are replaced by eukaryotic expression cassette in the recombinant AAV vector (rAAV). This cassette is usually composed of a promoter such as the CMV (CMVp) followed by the coding region for the gene and a SV40 polyadenylation signal sequence (pA).
Figure 1.10: Model of antibody gene expression in muscle. rAAV is used as a vehicle to carry the neutralizing antibody gene. After the virus vector is injected into skeletal muscle, the muscle cells will be transduced. The neutralizing antibodies will be produced and secreted into blood circulation.
CHAPTER 2

SCFV X5 ANTIBODY GENE TRANSFER INTO MICE
AND RHESUS MONKEYS

2.1 Introduction to our vaccine approach: “reverse immunization”

Our approach is predicated on the ability to achieve sustained, high concentrations of neutralizing antibodies in the systemic circulation by antibody gene transfer. Towards this goal, our laboratory has successfully demonstrated long-term expression of an anti-HIV-1 antibody (IgG1b12) in Rag-1 mice, following rAAV mediated delivery of heavy and light chain genes into mouse skeletal muscle (Lewis, Chen et al. 2002). Unfortunately, the observed expression level was below that predicted to be efficacious based on passive immunization studies (Mascola, Louder et al. 1997; Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999; Mascola, Stiegler et al. 2000; Mascola, Lewis et al. 2003). There are several possible explanations for the low expression levels observed. First, IgG1b12 is a large full-length IgG molecule with a molecular weight of 150 kD, which might prevent efficient secretion from an ecotopic site like skeletal muscle. Secondly, IgG is composed of two heavy chains and two light chains, and therefore, two different promoters were used to drive expression of each antibody chain. IgG concentration is thus limited to whichever polypeptide (heavy or light chain) is the most poorly expressed.
Herein, I have tested several approaches to optimize efficient antibody gene delivery in the Rag-1 mouse model to avoid the confounding variable of an active host immune response. A pilot experiment was also performed in the rhesus macaque model to determine whether rodent studies accurately predict NHP expression levels.

2.1.1 X5 – a novel anti-HIV NAb

X5 and IgG1b12 are two of the 5 broadly cross-reactive HIV-1-neutralizing human monoclonal antibodies identified to date. Both of them were originally obtained as Fab fragments by selection of phage display libraries from seropositive donors with relatively high broadly neutralizing serum antibody titers. Fab b12 was first isolated in early 1994 (Burton, Pyati et al. 1994), while Fab X5 was isolated in 2001 by panning against a gp120-CD4-CCR5 complex (Moulard, Phogat et al. 2002). Fab X5 binding to gp120 can be increased significantly in the presence of CD4 and weakly by addition of the CCR5 co-receptor.

Since their discovery, both of these molecules have been converted into different antibody forms such as scFv and complete IgG molecules. X5 recognizes a conserved CD4 inducible (CD4i) epitope on the gp120 surface and has been shown to possess in vitro neutralization activity against multiple primary HIV-1 isolates. It is the only broadly neutralizing antibody that exhibits increased binding to gp120 following HIV-1 attachment to the receptor/co-receptor complex. The CD4i epitope is formed after gp120 has attached to CD4 and is sterically hindered by the close proximity to the cell membrane. Therefore, the CD4i epitope is sterically restricted and short-lived. Fortunately, X5 features a 22 AA long CDR H3 hook-shaped motif, which is even longer than the b12 CDR3 loop. This motif facilitates binding to the recessed CD4i pocket in the gp120-CD4 complex (Saphire, Parren et al. 2001). This explains why its neutralization activity is
dramatically affected by steric constraints at the CD4i binding site (Labrijn, Poignard et al. 2003; Darbha, Phogat et al. 2004). Accordingly, the scFv X5 binds and neutralizes better than the Fab X5, which is in turn better than the whole IgG X5 molecule (Labrijn, Poignard et al. 2003). The breadth and potency of scFv X5 were further improved by random mutagenesis and sequential antigen panning to yield two potent mutants termed M6 and M9 (Zhang, Shu et al. 2004).

Due to difficulties in expressing high levels of IgG1b12, it seemed logical to explore whether the use of potent single-chain constructs (i.e., scFv X5) could result in increased NAb expression levels. In addition, we looked at several expression components to identify modifications in construct design that could lead to incremental improvements in expression levels. The first element we looked at was the signal peptide (SP).

2.1.2 Optimized leader peptides

The central hydrophobic region in the SP sequence is highly variable among different natural secretion proteins but rich in leucine, valine, and alanine (Valent, Kendall et al. 1995; Zheng and Gierasch 1996; Matoba and Ogrydziak 1998). The -1 and -3 positions of the cleavage site are usually small and neutral AA residues such as alanine, glycine, or serine (Nielsen, Engelbrecht et al. 1997). By computer modeling over 160 natural secretory SPs and experimental testing, Shi and colleagues identified several efficient artificial leader peptides (Barash, Wang et al. 2002). Two optimal SPs were identified termed synthetic leader peptide 1 (SL1: MWWRLWWLLLLLLLWPMVWA) and synthetic leader peptide 2 (SL2: MRPTWAWWFLVLLLALWAPARG). We hypothesized that these artificial leader peptides might facilitate improved scFv X5 secretion from transduced muscle cells, leading to increased NAb levels in vivo.
2.1.3 Self-complementary vectors.

Another possible option to increase antibody levels is to use second-generation self-complementary vectors. In the initial IgG1b12 expression studies, a standard single-stranded rAAV vector (SS rAAV) was used. As its name implies, SS rAAV vectors deliver a single-stranded DNA genome to the cell, which has to be converted by the host cell’s DNA synthesis machinery into a double-stranded DNA template for transcription. Several laboratories have demonstrated that second strand DNA synthesis is a rate-limiting step for rAAV mediated transgene expression (Ferrari, Samulski et al. 1996; Fisher, Gao et al. 1996).

To solve this problem, self-complementary AAV (SC rAAV) vectors were developed by Douglas McCarty and colleagues (McCarty, Monahan et al. 2001; Wang, Ma et al. 2003). The 5’ ITR region containing the terminal resolution site (TRS) was deleted to enable the efficient packaging of the single stranded dimeric replication form. Deletion of the 5’ TRS makes the dimeric replication form unable to be resolved into monomers, but it can still be packaged into AAV particles if the size of the construct is within packaging limits. Thus, the dimeric form is a hairpin that folds back onto itself when the virus uncoats within the nucleus. In addition to avoiding the requirement for second-strand DNA synthesis, the SC rAAV exhibits higher \textit{in vivo} DNA stability and more effective circularization than SS rAAV. These properties supply the basic molecular mechanisms for the more rapid and robust transgene expression observed with SC rAAV.

The efficiency of SC vectors has been tested in cell culture and in multiple \textit{in vivo} animal models (Fu, Muenzer et al. 2003; McCarty, Fu et al. 2003; Gao, Lu et al. 2006). SC rAAV2 expressing the enhanced green fluorescent protein (eGFP) efficiently transduced neurons and glial cells via
intravenous (IV) injection in mice (Fu, Muenzer et al. 2003). Tail vein injection of SC rAAV7 and SC rAAV8 encoding the rhesus beta subunit of choriogonadotropic hormone (bCG) demonstrated a 100 to 1000-fold increase in bCG expression relative to that observed after administration of the transgene by SS vectors in immunocompetent C57BL/6 mice. In cynomologus macaques, levels of transgene product achieved with SS AAV7 were below the detection limit at 6 weeks (< 50 relative units/ml), while SC AAV7 vector were 80 to 120-fold above the detection limit. Similar data were observed for SC rAAV8 vectors in non-human primates (Gao, Lu et al. 2006). A similar result was also obtained for human factor IX (hF.IX) following rAAV8 mediated gene transfer to the liver with an SC vector (Nathwani, Gray et al. 2006). Because of these pre-clinical studies, a SC rAAV vector encoding hF.IX was used in a recent clinical trial (Wu, Sun et al. 2008). The principle drawback with SC vectors is the packaging size of the transgene which is limited to ~ 2.2 kb. Fortunately, the scFv expression cassettes (~ 2.0 kb) are small enough to be incorporated into SC rAAV vectors. Interestingly, the packaging limit of the SC vector was extended to about 3.3 kb by reducing levels of Rep protein expression in an AAV helper plasmid (Wu, Zhao et al. 2007). It is theorized that when the packaged DNA genome is larger than a certain limit, high levels of Rep expression might cleave the DNA at putative terminal resolution-like sites in the rAAV genomes prohibiting larger DNA molecules from being encapsidated.

2.1.4 Viral dose saturation

Vector dose per injection site in the muscle tissue is another possible factor that needs to be considered for optimization of transgene expression. Studies in tissue culture using human myotubes to express hF.IX demonstrated that the capacity of skeletal muscle to synthesize biologically active hF.IX was saturable (Arruda, Hagstrom et al. 2001). Moreover, using a
hemophilia B dog model, the same research group demonstrated that rAAV2 mediated gene transfer of canine factor IX (cF.IX) can be as high as $2 \times 10^{12}$ vg per injection site before initiation of a significant immune response (Herzog, Fields et al. 2002). Additionally, it was demonstrated by our laboratory in mouse muscle and human tissue samples that rAAV predominately exists as circular double-stranded episomes (Schnepp, Clark et al. 2003). The circularization of the DNA vector is dependent on host cellular enzymes such as DNA-dependent protein kinase (DNA-PK) (Sanlioglu, Duan et al. 1999; Song, Laipis et al. 2001; Wang 2002). Therefore, at very high intranuclear copy number of rAAV genomes per cell may saturate cellular circularization pathways. High vector inputs could result in strand annealing that can lead to transient gene expression off of this linear form. Moreover, if extremely high protein synthesis occurs due to dose saturation, it might conceivably induce an ER stress response due to accumulation of misfolded proteins in the ER lumen (Rao, Hermel et al. 2001). To address the possibility of dose saturation, we first determined whether dose saturation was occurring locally when we injected rAAV1/CMV.scFvX5 in muscle if the same dose was given into a single muscle group or multiple muscle groups. In other words, can we achieve higher serum scFv X5 concentration if we split one dose into multiple doses for intramuscular injection?

2.2 Materials and methods

2.2.1 Clone scFv X5 into SS rAAV

All of DNA manipulations were performed using standard techniques (Sambrook, Maniatis et al. 1989). X5scFv/pCombX, a phage plasmid (4.08 kb) containing the gene for scFv X5 (822 bp), was a gift from Dr. Dennis R. Burton (Scripps Research Institute, CA). Three forward primers, containing either the optimized leader sequence SL1, SL2, or the control natural IgG leader sequence (NL), were designed. For-SL1 (104-mer), the sequence is: 5’- CTT AGC GGC GGC CGC
CCA CCA TGT GGT GGC GCC TGT GGT GGC TGC TGC TGC TGC TGC TGC TGC TGT GGT CCA TGG TGT GGG CCG ATA TTG TGC TGA CGC AGT CTC CA-3’; For-SL2 (110-mer), the sequence is: 5’- CTT AGC GGC CGC CCA CCA TGC GCC CCA CCT GGG CCT GGT GGC TGT TCC TGG TGC TGC TGG CCC TGT GGG CCC CCG CCC GCG GCG ATA TTG TGC TGA CGC AGT CTC CA-3’; For-NL (41-mer), the sequence is: 5’- CTT AGC GGC CGC CCA CCA TGA AAA AGA CAG CTA TCG CGA TT-3’). The reverse primer paired with all three forward primers is X5Rev (5’-CTT AGC GGC CGC CTC ATG AGG AGA CGG TGA CCA GGG TTC CCT-3’). The For-SL1 and For-SL2 oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE). Both forward and reverse primers contain a Not I site for cloning. The three 0.9 kb fragments containing the scFv X5 gene were amplified by standard PCR, digested with Not I, and inserted into the plasmid of pAAV/CMV.β-gal/c.13 containing a SV40 polyadenylation site (Clark, Voulgaropoulou et al. 1995). Resulting clones were sequenced using an ABI 3100 automated sequencer by the DNA Sequencing Core Facility at The Research Institute at Nationwide Children’s Hospital. Thus, we generated three pAAV/CMV.scFv X5 vector plasmids with different leader sequences (Figure 2.1). All the plasmids were amplified in E.coli DH5alpha cells and purified by QIAGEN Endotoxin-free kits (QIAGEN Inc., CA).

2.2.2 Cells
HeLa (ATCC product number CRL-2) and C2C12 cells (ATCC product number CRL-1772) were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Cosmic Calf Serum (CCS) with 5.0 μg/ml Ciprofloxacin Hydrochloride (CIP) (ICN Biomedicals, INC., Ohio). Low passage HeLa cells, a human cell line from cervical cancer cells, were used to make the stable cell lines as described in the following section. All stable producer
cell lines were maintained in the 10% CCS containing DMEM medium with G418 at a final concentration of 700 μg/ml.

2.2.3 Construction of producer cell lines for SC rAAV1/CMV.scFvX5 production

Starting with plasmid pAAV/CMV.scFvX5 as a template, the CMV-X5-polyA cassette (1.86 kb) was amplified by PCR using the following two primers: CMVstart: 5′-CGG TTT AAA CTC GTT ACA TAA CTT ACG GTA AAT GGC-3′; PolyAend: 5′-CCC ACT AGT GAA AAA ACC TCC CAC ACC-3′. Pme I and Spe I restriction sites were inserted into the two primers to facilitate the cloning process. The fragment was isolated from a 0.8% DNA agarose gel, digested with the two restriction enzymes, and then inserted into a SC AAV cloning vector called pHpa7. This is a cloning vector from Dr. Douglas McCarty that has Hpa I and Xba I cloning sites to insert the CMV-X5-polyA cassette between two AAV ITRs (one of which lacks the terminal resolution site). This process generated plasmid SC pAAV/CMV.scFvX5. To generate a tripartite producer plasmid for stable cell line production, a Swa I site in the pHa7 plasmid backbone was used to insert a Cap1-Rep2-NeoTK fragment (5.95 kb) by blunt end ligation to generate pAAV/CMV.scFv X5.Cap1-Rep2-NeoTK. Orientations of the fragment in the plasmids were screened and compared for the efficiency of the production of the virus by transient transfection in 293T cells.

In order to make sufficient amounts of virus for mouse and subsequent rhesus macaque studies, stable producer cell lines were isolated based on the protocol previously developed in our laboratory with minor modifications (Clark, Voulgaropoulou et al. 1995). Briefly, 2 μg of plasmid pAAV/CMV.scFv X5.Cap1-Rep2-NeoTK was used to transient transfect low pass HeLa cells in a 6-well plate by using FuGENE 6 (Roche Applied Science, Cat. #1814443) with
subsequent G418 (700 μg/ml) drug selection in a T175 flask. After the cells grew to 75 - 90% confluency, the cells were trypsinized and re-seeded in 100 mm cell culture dishes at different cell densities. After single cell colonies formed in these dishes, about 1000 individual cell colonies were manually picked into 96 well plates and screened following Ad5 infection (multiplicity of infection [MOI] = 20). Cell line L21021 was identified as the best producing cell line (2.5x10^5 DRP/cell).

2.2.4 Virus production and purification

SC rAAV1 vectors were produced by infecting L21021 with wild-type Ad5 (MOI = 20) using a Corning Cell Cube adherent bioreactor. The producer cell line was first expanded in DMEM in T175 flasks supplemented with 10% CCS, 1% PenStrep, and 700 μg/ml G418. A mini-cell cube (Corning Incorporated, Cat. #3101) was seeded with 2x10^8 cells in 1.6 liters of 10% DMEM medium and, after 6 days growth, the cells were infected with Ad5 at an moi = 40. Three days after infection, the cells were harvested for downstream purification.

Virus purification was performed by modified methods of published (Clark, Liu et al. 1999; Zolotukhin, Byrne et al. 1999; Zolotukhin, Potter et al. 2002). Briefly, Ad5 infected L21021 cells were re-suspended in TMN200 (20 mM Tris-HCl, 1 mM MgCl₂, 200 mM NaCl, pH 8.0) at a concentration of 5x10^6 cell/ml in 500 ml sterile centrifuge bottles, followed by 4 freeze/thaw cycles to lyse the cells. The cell suspension was vortexed at the maximal speed for at least 1 min. after each thaw. Benzonase (Sigma, Cat. #E1014) was then added to the cell lysate at a final concentration of 35 U/ml and incubated at 37°C water bath for 30 min. to reduce viscosity. The crude lysate was then clarified by centrifugation at 3,500g for 30 min. The Ad5 was inactivated by heating the lysate in a 56°C water bath for 30 min., followed by centrifugation, and the
supernatant was frozen at -80°C. Iodixanol gradients were then used to purify the virus. Iodixanol step gradients were formed in Optiseal tubes (Beckman, Cat. #361625). Gradients were formed using 15 ml of clarified lysate, followed by under laying with stepped iodixanol density gradients (5, 5′-[2-hydroxy-1, 3-propanediyl] bis(acetyl-anino)] bis[N, N′-bis(2,3-dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide] prepared in PBS-MK buffer (1xPBS, 1 mM MgCl₂, and 2.5 mM KCl). Each gradient was added in the following order: 6 ml 15%, 5 ml 25%, 3 ml 40%, and final 2 ml 60% iodixanol. The 15% density gradient also contained 1.0 M NaCl to prevent virus aggregation. For purposes of distinguishing each gradient, a small amount of neutral red was added to the 25% and 60% gradients. The tubes were centrifuged in a 70 Ti rotor at 80,000 rpm for 1 hour at 22°C. Approximately 3 ml of the 40% gradient was collected after side puncture and pooled for final column chromatography.

The pooled AAV-containing fraction was diluted with 5 volumes of TM buffer (10 mM Tris-HCl, 1 mM MgCl₂, pH 8.0) and centrifuged at 3,500 rpm for 30 min. at 4°C. The supernatant was filtered through a 0.45 μm filter, and the diluted material was loaded onto a pre-equilibrated POROS HQ-50 column (20 mM Tris-HCl, 1 mM KCl, 35 mM NaCl, pH 8.0). The column was then washed extensively with a low salt wash (20 mM Tris-HCl, 40 mM NaCl, pH 8.0) until the OD280nm reached baseline. The virus was eluted with a NaCl salt gradient from 40 mM to 1,200 mM in 15 column volumes (CVs). The virus eluted at approximately 200 mM NaCl. Peak and side fractions (1 ml) were collected by an automatic sample collector and the amount of virus in each tube was determined by the Q-PCR DNase resistant particle (DRP) analysis. The purity of each fraction was checked by SDS-PAGE analysis. Fractions containing highly pure virus were pooled together and the virus was dialyzed in a storage buffer (100 mM Sodium Citrate, 10 mM Tris-HCl, pH 8.0) using a Slide-A-Lyzer cassette (3.0 – 15.0 ml, MWCO = 10k, Pierce, Cat.
A non-ionic detergent (Pluronic F68) was added to the final preparation at a concentration of 0.001% to prevent loss of virions to surfaces during handling (Wright, Qu et al. 2003). Lastly, the purified vector preparation was sterile filtered (0.22 μM, Fisher, Cat. #SLGV004SL) and a final titer determined by Q-PCR DRP analysis.

DRP in the crude lysate and purified virus were determined by real-time Q-PCR as previously detailed (Clark et al., 1999). This method quantitates encapsidated DNA particles by extrapolation from a plasmid standard curve (using system software from ABI). The standard curve was generated from samples in triplicate using a serial dilution of 10^{11} copies/ml plasmid stock on the same plate (coefficient of linearity, ≥ 0.95).

### 2.2.5 Animals

Mice and rhesus macaques were maintained in accordance with Nationwide Children’s Hospital Institutional Animal Care and Use Committee. Six to eight-week-old Rag-1 mice were used for our preliminary experiments. The mice were bred in our Research Vivarium Facility and the breeders were purchased from Jackson Laboratory (C.129s7 (B6)-Rag-1^{tm1Mom}, Bar Harbor, Maine). Intraperitoneal injection of 2,2,2-tribromoethanol was used to anesthetize the mice (concentration 240 mg/kg). Fifty μl of virus solution (diluted in PBS if needed) was injected into the quadriceps femoris or gastrocnemius muscle along the long axis of the muscle using a 28-gauge needle. Serum samples (100 – 200 μl) were collected from the retro-orbital sinus under anesthesia. At the time of sacrifice, the entire muscle was cut and bisected along the transverse plane. Half of the muscle was fixed in a non-cross-linking fixative (Histochoice; Amresco, Solon, Ohio) and embed in paraffin, while the other half of the muscle was saved for later DNA or
protein extraction. The muscles from the same position in control mice and the un-injected legs were taken as control tissues.

Total soluble muscle proteins were extracted using a tissue specific protein extraction buffer (T-PEB, Pierce Biotechnology Company, Cat. #78510) following their standard protocol. Briefly, freshly thawed tissue (~ 40 mg) was frozen using liquid nitrogen and ground quickly into a powder using a mortar and pestle. The powder was then transferred into a pre-chilled micro-homogenizer filled with 0.5 ml T-PEB containing complete protease inhibitor cocktail (Roche, Cat. #11697498001, 50 ml of extraction buffer/tablet). After the mixture was homogenized 20 to 30 times on ice, the muscle homogenate was transferred to a 1.5 ml micro-centrifuge tube and spun for 10 min. at 12,000 rpm to pellet the tissue debris at 4°C. The protein-containing supernatant was then carefully transferred to another chilled 1.5 ml micro-centrifuge tube. The samples were frozen at -80°C for later concentration measurements. Total protein concentration in each supernatant was measured using a BCA protein assay kit (Pierce, Cat. #23252) according to the manufacture’s standard protocol. The scFv X5 concentration in each supernatant was then measured using a modified ELISA method detailed in Section 2.2.5. The total amount of protein scFv X5 in the muscle was calculated according to the size of the muscle and the volume of the extraction buffer used.

Chinese rhesus macaques (Macaca mulatta) were used in a pilot study. Animals were pre-screened by ELISA to ensure that the monkeys had low-levels of anti-AAV binding antibody prior to rAAV injection. Two monkeys (RQ6498-M1 and RQ6517-M2) received intramuscular injections into both quadricepses at a total dose of 3.5x10^{13} DRP of the SC rAAV1/CMV.scFv X5 vector per monkey. Each injection contained 1.5 ml volume of the vector and one injection
site was performed per leg. During the injection procedure, animals were anesthetized with Telazol (4 mg/kg). Telazol is a rapidly acting general anesthetic drug that provides significant pain relief, immobility, and muscle relaxation. The animals were bled biweekly and the concentrations of scFv X5 were measured by a gp120 ELISA (Section 2.2.5). The two animals as well as four naïve monkeys (RQ6623, RQ6628, RQ6637, and RQ6647) were challenged after one year and followed for another year. Animals were euthanized in accordance with the Nationwide Children’s Hospital Research Institute Vivarium Protocol.

2.2.6 Challenge virus, procedure, and virus load measurement

The challenge virus used in this pilot study was SHIV_{SF162P3} passage 3 from NIH AIDS Research & Reference Reagent Program (Cat. #6526). The virus cloning and isolation process had been described elsewhere (Harouse, Gettie et al. 1999; Harouse, Gettie et al. 2001). Briefly, SHIV_{SF162} was constructed by replacing the tat, rev, and env genes of the molecular clone SIVmac239 with corresponding regions from the R5-tropic, HIV-1 molecular clone SF162 (Tan, Harouse et al. 1999). SHIV_{SF162P3} was obtained from SHIV_{SF162} by serial passaging of the virus in rhesus monkeys. Virus was re-isolated and re-transfused into blood-bone marrow in each passage. SHIV_{SF162P3} retains the R5 phenotype of the original SHIV_{SF162} (Harouse, Gettie et al. 1999).

Rhesus macaques were challenged by the intra-rectal route with 50 MID_{50} of SHIV_{SF162P3}. The animals were anesthetized by using intramuscular injection of Telazol (4 mg/kg) that kept them immobile for at least 1 hour. Each animal was then laid in a prone position with the rump elevated by a roll-type cushion so that the rectal vault was canted downward. A 2 mm suction catheter was gently inserted into each monkey’s rectum, and challenge virus slowly instilled...
through the catheter into the rectum. The animals were left in the prone position for 5 min. after the rectal instillation of the virus and then moved back to their cage.

Viral load was measured using the VERSANT bDNA 3.0 assay kit (Bayer Corp., Diagnostics Division, Tarrytown, N.Y.). Viral load measures the number of copies of viral RNA per milliliter of plasma. The bDNA (branched DNA) method is a hybridization-based methodology (Tsongalis 2006) (Figure 2.1). Briefly, virus was collected from monkey plasma by ultra-centrifugation and was digested with proteinase K to ensure that the viral RNA sample was free of inhibitors and available for hybridization. Target-specific oligonucleotide probes (label extenders and capture extenders) were then hybridized at high stringency to the viral RNA. Capture extenders were designed to hybridize to the viral RNA and capture probes, which were immobilized on a micro well plate before hand. Label extenders were designed to hybridize to the regions next to the capture extenders and to provide sequences to hybridize to a preamplifier oligonucleotide. The preamplifier was used to hybridize to multiple amplifier molecules that created a bDNA structure. Finally, alkaline-phosphatase- (AP) labeled oligonucleotide probes were bound to the bDNA molecule. A chemiluminescent product of the AP mediated reaction was then detected as the bDNA signal. This signal was proportional to the number of AP-labeled probes that hybridized to bDNA secondary sequences, which are ultimately proportional to the number of viral particles. Absolute quantification was obtained by establishing a standard curve for each run.

2.2.7 Enzyme immunoassay

The scFv X5 levels in cell culture medium were detected by a published ELISA method developed in the Burton laboratory with some minor modifications to accommodate detecting scFv X5 instead of Fab X5 (Moulard, 2002). Briefly, HeLa and C2C12 cells (3x10^5/well) were
co-transfected with 2 μg of plasmid pAAV/CMV.scFv X5 and pAAV/CMV.β-gal at 1:1 ratio in a 6-well plate using FuGENE 6 reagent (Roche Applied Science, Cat. #1814443) according to the manufacturer’s guidance. Construction of pAAV/CMV.β-gal was previously reported by our laboratory (Clark, 1995). After 48 hours, the cell supernatant was removed (and saved) and the cells lysed with 250 μl M-PER buffer (Pierce, Cat. #78501) at room temperature for 5 minutes with gentle shaking. After cell lysis, a β-gal assay reagent (Pierce Chemical All-in-One β-gal reagent) was added to each well and incubated at 37°C for 30 minutes. The OD405nm absorbance was measured and this value used to normalize for the transfection efficiency. To measure the relative levels of scFv X5 in the cell supernatant, 96-well ELISA plates were coated with 100 ng/well Bal gp120 (NIH AIDS Research & Reference Reagent Program, Cat. #4961) in 50 μl volume of PBS buffer overnight at 4°C. The plate was then blocked with 100 μl of 3% BSA/PBS (blocking buffer) for 1 hour at 25°C. After five PBST (PBS containing 0.05% Tween 20) washes, wells were incubated with 50 μl of diluted cell culture supernatant for 1 hour at 25°C. After 10 washes with PBST, 50 μl of a 1:500 dilution of protein L-HRP conjugate in blocking buffer (Pierce, Cat. #32420) was added and incubated for 1 hour at 25°C. After 10 more washes with PBST, the assay was developed with 3,3’, 5,5’-Tetramethylbenzidine (TMB) liquid substrate (Sigma, Cat. #T8665-100ML) for 30 min. at 25°C. The reaction was stopped by adding 100 μl 1N H₂SO₄ to each well and the data collected using an ELISA plate reader measuring the optical density at 450 nm.

The scFv X5 levels in animal sera were measured using a modified ELISA based on the above detection protocol. Briefly, Bal gp120 and sCD4 (NIH AIDS Research & Reference Reagent Program, Cat. #7356) were used to coat 96 well ELISA plates (100 ng Bal gp120, 50 ng sCD4/well) in PBS buffer at 4°C overnight. The following morning, each well was blocked with
285 μl of 3% BSA in PBST (blocking buffer) for 2 hours with constant shaking at 25°C. After 4 washes with PBST again, the wells were incubated with 100 μl of diluted serum samples and scFv X5 standards (a gift from Dr. Dennis Burton) in PBST for 1 hour at 25°C with constant shaking. The protein L-HRP conjugate was used as the secondary antibody at a 1:250 dilution. After four washes with PBST, 100 μl of the diluted protein L in blocking buffer was added to each well and incubated at 25°C for 2 hours. After 4 PBST washes, the plate was developed using One-Step Ultra-TMB reagent (Pierce, Cat. #34028) for one hour at 37°C. The reaction was stopped with 100 μl 1N H₂SO₄. The serum scFv X5 concentrations were calculated according to a standard curve using purified X5 of known concentration (coefficient of linearity, ≥ 0.99).

Detection of anti-AAV antibodies was performed by a modified ELISA method developed in our laboratory. Briefly, 96 well ELISA plates were coated with 100 μl/well of rAAV1 vector at a concentration of 2x10¹⁰ DRP/ml in a carbonate buffer (0.2 M Sodium carbonate-bicarbonate, pH 9.4) at 4°C overnight. The plate was then blocked with blocking solution (5% nonfat dry milk, 1% normal goat serum in PBS buffer) at 37°C for one hour with constant shaking. The wells were then incubated with 100 μl diluted animal serum (1:100 dilution in the blocking solution) for one hour at 25°C. After 5 PBST washes, the wells were incubated with a goat anti-monkey IgG HRP conjugated antibody (Sigma, Cat. #A2454) diluted in the blocking solution (1:10,000) in a volume of 100 μl for 30 min. at 25°C. After washing the plate 5 times with PBST, the plate was developed with 100 μl One-Step Ultra-TMB substrate for 10 min. at 25°C. The reaction was stopped by adding 100 μl 1N H₂SO₄. Sample absorbance was read at OD450nm and samples in which antigen coated wells had an OD450nm value 2 fold higher than non-antigen coated wells were considered positive at that dilution.
2.2.8 HIV neutralization assay

Neutralization of HIV-1 was measured by a reduction of Tat-regulated luciferase reporter gene expression after a single-cycle infection of the engineered HeLa cell line TZM-bl using different HIV-1 pseudoviruses, as previously described (Platt, Wehrly et al. 1998; Wei, Decker et al. 2002) (Figure 2.2). The experiments were performed in Dr. David Montefiori’s laboratory at Duke University. The TZM-bl cell line (also called JC57BL-13) expresses CD4, CXCR4, and CCR5 and contains integrated firefly luciferase and β-gal reporter genes under the control of the HIV-1 LTR (Figure 2.2: A). The neutralization assay consists of three steps: 1) preparation of pseudotype viruses (Figure 2.2: B); 2) infection of TZM-bl cells (Figure 2.2: C); and 3) measurement of the luciferase activity. Briefly, pseudoviruses were prepared by co-transfecting 293T cells with an Env expressing plasmid and a second plasmid that expresses the entire HIV-1 genome except Env. The pseudovirus particles are infectious but cannot generate infectious progeny virus for next round infection due to the lack of the Env gene in the virus genome. The serum samples were heated to 56°C for 1 hour to inactivate complement before neutralization measurement. Complement proteins in the serum of rhesus monkeys may lyse the cell as well as HIV-1 virus and cause high assay background (Montefiori 1997). TZM-bl cells were then infected with a particular HIV-1 pseudovirus in the presence of different dilutions of monkey serum. After 48-hour incubation at 37°C in a CO₂ incubator, the cells were harvested for luciferase luminescence measurement using the Bright Glo Luciferase Assay Kit (Promega, Cat. #E2620). The results were expressed as TCID₅₀: the value of the serum dilution at which the relative luminescence was reduced by 50% compared to virus control wells incubated with naïve control monkey serum.
2.2.9 Western blot

Western blot analysis was used to detect cellular expression levels of scFv X5. Briefly, HeLa cells (3x10^5/well) were transfected with 2 μg of plasmid pAAV/CMV.scFv X5 in a 6 well plate. After 48 hours, both cell supernatant and cells were harvested. Cell supernatant was diluted 1:1 with 2x SDS loading buffer (2 ml glycerol, 4 ml 10% SDS, 4 mg bromophenol blue, 2.5 ml 50 mM Tris-HCl buffer, pH 6.8, 1.5 ml ddH2O). The cell pellet was re-suspended in 200 μl of 1x SDS loading buffer. Samples were boiled for 5 min. and 20 μl of each sample was loaded onto a 10% SDS-PAGE gel. The gel was run at 100 Volts (V) for 2.5 hours and proteins were transferred to a pre-wetted Hybond-P membrane (Amersham Biosciences, Cat. #RPN2020F). The membrane was blocked with 5% nonfat dry milk in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, 0.1% Tween 20) after transfer. The membrane was incubated with HRP conjugated Protein L at a 1:10,000 dilution. Immuno-detection was achieved using the ECL Plus Western Blotting Detection System according to the manufacturer’s instructions (Amersham Pharmacia, Cat. #RPN2132).

Antibodies against scFv X5 in animal’s serum were detected by a modified traditional western blot approach called a western strip blot. Briefly, 1.0 μg purified scFv X5 from cell culture was run on a 10% SDS-page gel in a single large well. After transferring to a Hybond-P membrane, the protein was subjected to western strip blot hybridization by inserting the membrane into a strip-lane manifold to obtain discrete lanes for primary antibody (diluted monkey sera) incubation. Following incubation with monkey sera (1:100 dilution) in 5% milk in TBST, the blot was incubated with a goat anti-human IgG-HRP conjugate (Fc specific, Sigma, Cat. # A0170-1ml) at a 1:10,000 dilution. Detection was visualized using the ECL system.
2.2.10 Monkey IFN-\(\gamma\) ELISpot assay

Sets of overlapping peptides encompassing the AAV1 VP1 capsid protein and eGFP (18 amino acids overlapping by 11 residues) were synthesized and purified by Genemed Synthesis, Inc. (San Francisco, CA). Each peptide was supplied as a 10 mg/tube lyophilized powder. Upon arrival, the peptide was dissolved in 200 \(\mu\)l DMSO, vortexed until dissolved, and followed by adding 1.8 ml sterile water to yield a final concentration of 5 mg/ml. Peptide pools were made by combining different peptides together. For eGFP, only one pool (Pe) was made by combining all 33 peptides at a final concentration of 40 \(\mu\)g/ml for each peptide. For VP1, three peptide pools were made according to the protein sequence of VP1. Pool 1 (P1) included peptides 1 to 35, pool 2 (P2) 36 to 70 and pool 3 (P3) 71 to 104. The final concentration of each peptide in P1, P2, and P3 was also 40 \(\mu\)g/ml for each peptide.

IFN-\(\gamma\) ELISpot assay was used to detect capsid-specific gamma interferon (IFN-\(\gamma\)) secretion (if present). The experiment was performed using the monkey IFN-\(\gamma\) ELISpot kit (U-CyTech, Netherlands, Cat. #CT126-PB5) according to the manufacturer’s instructions. Briefly, thawed PBMCs, isolated by Ficoll-Pacque density gradient centrifugation, were washed in PBS, counted, and re-suspended in AIM-V medium (Gibco, Cat. #12055) containing 2% human serum (Sigma, Cat. #H1388) and 1x Pen/Strep (Gibco-BRL, Cat. #15140-122). Then the cells were incubated in a 37°C 5% CO\(_2\) incubator with an anti-IFN-\(\gamma\) capture antibody (MD-1) coated 96-well PVDF membrane-bottom plate at a cell density of 4x10\(^5\) cells/well with either Concanavalin A (Sigma, Cat. #C0412) at 10 \(\mu\)g/well or peptide pool at 0.4 \(\mu\)g/well. ConA was used as a lymphocyte mitogen which stimulated lymphocytes to grow and served as the assay positive control. After a 36-hour incubation, the plate was washed with PBST five times and incubated with 100 \(\mu\)l of a diluted biotinylated detection antibody for 1 hour at 37°C. After extensive washing with PBST,
the plate was incubated with 50 μl/well of a GABA (labeled goat anti-biotin antibodies) solution for 1 hour at 37°C. Spots were developed by incubating the plate with 30 μl/well of Activator I/II solution (substrate for spot formation supplied in the kit) for 15 to 30 minutes. After clear spots developed, the reaction was stopped by rinsing the wells with demineralized water. Numbers of spots were counted by an ImmunoSpot S5 Macro Analyzer (CTL Analyzer LLC, Cleveland, Ohio) after the plate was dried. The number of the spots forming cells (SFC) was normalized to 1x10⁶ PBMC. A well that had more than 50 SFCs/1x10⁶ cells above the negative control well was considered positive (Tobery and Caulfield 2004).

2.3 Results

2.3.1 Leader peptides, scFv X5 antibody, and SC rAAV vectors for antibody expression

Passive immunization studies with potent broadly NAbs have clearly shown that the concentration of the NAb is a critical factor in achieving a therapeutic effect in animal models (Mascola, Louder et al. 1997; Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999; Mascola, Stiegler et al. 2000; Mascola, Lewis et al. 2003). These studies argued that plasma NAb levels between 50 - 200 μg/ml at the time of virus challenge may in fact be sufficient to provide complete protection in a significant proportion of vaccinees, and result in reduced viremia and increased CD4+ levels in those that do become infected. Our previous studies with rAAV2/CMV.IgGlb12 yielded levels of IgGlb12 that were at least 10-fold below experimental levels that demonstrated efficacy. Thus, we needed to explore ways to increase antibody expression. We initially evaluated 3 design elements for the ability to increase antibody expression. First, we compared native and synthetic leader peptides. Secondly, we explored the utility of a single polypeptide (i.e., scFv X5) to determine if antibody levels were improved
compared to the expression of a full-length immunoglobulin molecule that is composed of 2 polypeptides. Finally, we determined whether a self-complementary vector could increase in vivo expression levels.

2.3.1.1 Evaluation of alternative leader peptides for enhanced secretion

The synthetic leader peptides SL1 and SL2 were predicted to be the two best leader sequences among 182 sequences previously tested (Barash, Wang et al. 2002). To determine whether these two artificial leader peptides could facilitate scFv X5 secretion from muscle cells, I cloned the scFv X5 gene into a pAAV vector controlled by the CMV promoter containing one of three different signal peptides (a natural IgG leader (NL), SL1, and SL2) (Figure 2.3). After transient transfection of HeLa cells with the above pAAV plasmids for 48 hours, the scFv X5 antibody levels in the cell supernatant and pellet were detected by western blot (semi-quantitative). As Figure 2.4 shows, there was a dramatic difference in the cellular export of scFv X5 upon leader peptide substitution with both synthetic leader sequences. With the NL sequence, the cell pellet fraction contained the vast majority of the scFv X5 protein (lane NL-CP) with very little secretion into the supernatant (lane NL-Sup). Thus, most of the scFv X5 protein was retained inside of the cells with the NL sequence. In sharp contrast, for both SL1 and SL2 leader sequences, the cell pellet had much less scFv X5 protein (lanes SL1-CP and SL2-CP) than that found in the supernatant (lanes SL1-Sup and SL2-Sup). These data clearly showed that the synthetic leader peptides SL1 and SL2 mediated much better secretion of the scFv X5 protein than the natural leader sequence. To measure the difference in secretion of scFv X5 semi-quantitatively and to find out whether better secretion is also observed in a myoblast cell line, a similar transient transfection experiment was performed using HeLa and C2C12 cells. The HeLa cell is an immortal human cervical cancer cell line, while C2C12 cells were originally derived from mouse
thigh muscle. In addition to plasmids containing scFv X5, a \( \beta \)-gal expression plasmid with a similar size to the pAAV/CMV.scFv X5 plasmid was co-transfected in the above cells at a 1:1 ratio in a 6-well format. After 48 hours post-transfection, both the cell supernatant and cell pellet were harvested. The cell supernatant was used to measure the secreted scFv X5 levels by an ELISA method. The cell pellet was used to measure \( \beta \)-gal levels inside of the cells to normalize for DNA transfection efficiency using a \( \beta \)-gal Assay Kit (detail in materials and methods). As Figure 2.5 shows, in HeLa cells, cell supernatant from the X5 plasmid containing the SL1 leader sequence obtained a higher OD450nm value diluted 1:100 compared to undiluted X5 possessing the native leader. This result suggested that the SL1 sequence mediates significantly better secretion efficiency than the plasmid containing the NL sequence. Similarly, X5 containing the SL2 also gave comparable secretion to the SL1 leader. In C2C12 cells, the plasmid containing SL1 sequence was about 20-fold higher than that from the plasmid containing the NL sequence. In addition, the OD450nm value from the plasmid containing the SL1 sequence was 75% higher than that from the plasmid containing the SL2 sequence. These data demonstrated that scFv X5 can be expressed in the muscle cells and the synthetic leader sequences mediate higher secretion efficiency than NL in both HeLa and C2C12 cells. Therefore, these data provide the rationale for using synthetic leader sequence SL1 to increase in vivo secretion efficiency.

2.3.1.2 SC vs. SS vectors

Self-complementary (SC) vectors can improve protein expression dramatically by avoiding the requirement for second-strand synthesis (McCarty, Monahan et al. 2001; Fu, Muenzer et al. 2003). To directly test whether a SC vector could increase scFv X5 expression levels, I PCR amplified the scFv X5 expression cassette (CMV promoter, SL1 leader peptide, scFv X5, and
polyadenylation site) and cloned this fragment into a SC base vector termed pHpa7 using Hpa I and Xba I restriction sites. The cloning process is outlined in Figure 2.6.

For large-scale production of the SC rAAV1 virus, a tripartite producer plasmid (pAAV1/CMV.scFv X5.Cap1-Rep-Neotk) was constructed by inserting the Cap1-Rep2-Neotk fragment into the SC pAAV/CMV.scFv X5 plasmid (Figure 2.6). The producer plasmid was transfected into low passage HeLa cells and screened for high-titer production following Ad5 infection and dot blot hybridization to detect vector replication. Subsequent Q-PCR analysis of productivity by measuring DRP was used to identify a superior cell line (L21021). Large-scale vector production was achieved by infecting L21021 cells with Ad5 (moi = 40) in a Corning Cell Cube adherent bioreactor. The cells were harvested 3 days later after adenovirus cytopathic effect and vector purified by iodixanol gradient and ion exchange chromatography. Purity was assessed using SDS-page (Figure 2.7). The SS rAAV1/CMV.scFv X5 vector was produced and purified using similar methods by Targeted Genetics Corporation (Seattle, WA).

To compare the \textit{in vivo} expression levels of SC vs. SS rAAV1/CMV.scFv X5 vectors, we intramuscularly injected 6-week old immunodeficient Rag-1 mice at a dose of $1.3 \times 10^{11}$ DRP/animal. The mice were bled biweekly and the scFv X5 serum concentrations were measured by a gp120 binding ELISA using a scFv X5 protein standard provided by Dr. Denis Burton. As Figure 2.8 shows, the scFv X5 serum levels with the SC vectors were significantly higher than the SS vectors in all mice from weeks 2 to 16. On average, scFv X5 serum concentrations from the SC vectors ranged from 0.7 to 3.8 μg/ml while that from the SS vectors ranged from 0.05 to 0.5 μg/ml (p < 0.05). These data demonstrated 3 - 14 fold higher scFv X5 levels using a SC vector.
compared to the standard SS vector over all time points (2 -16 weeks). These data support the use of SC vectors to achieve increased scFv X5 levels in serum.

2.3.2 Multiple injections vs. single injection

At the dose utilized in our rodent experiments (≈ 5x10^{12} DRP/kg), it is possible that the muscle tissue near the injection site might be saturated with virus particles, which could have several negative consequences as I discussed in the introduction. Therefore, I asked whether multiple injections could allow for increased antibody production compared to a single bolus injection. The concept is that by achieving a greater tissue distribution of the vector, increased Ab levels could be realized in the circulation. To test this, I compared 1, 2 and 4 injections at different sites in the muscle, keeping the total dose constant (10^{11} DRP/animal). The single injection was given to the left quadriceps muscle. The two injections were performed on both the quadriceps and gastrocnemius muscle of the left leg. For animals receiving four injections, the quadriceps and gastrocnemius muscles in both legs were used. If vector saturation is occurring with a single injection, then 2 or 4 injections should produce greater amounts of scFv X5. Single injections produced scFv X5 in the range of 0.1 to 0.6 μg/ml which is consistent with the previous SS vector injections with a similar virus dose. The single and double injections produced similar amounts of scFv X5 protein in later weeks (weeks 8, 12, and 16), while 4 injections produced slightly less protein at weeks 2, 4, and 8 (Figure 2.9). Statistically, the scFv X5 levels from single injection did not differ when compared to levels from 2 and 4 injections (p > 0.05) except at weeks 2 and 4 (p < 0.05). The scFv X5 levels from two and four injections did not achieve a significant difference at all the weeks tested (p > 0.05) except at week 8 (p < 0.05). Therefore, direct intramuscular injection at 5x10^{12} DRP/kg in a small volume did not appear to saturate the targeted muscle site.
This bodes well for high dose rAAV administration to one or a few intramuscular sites at the level tested here.

Since dose saturation was not observed at 5x10^{12} DRP/kg, we asked whether high-dose administration could increase levels in a linear fashion. Mice were dosed with 6x10^{12} DRP (3x10^{14} DRP/kg) of SS or 10^{12} DRP (5x10^{13} DRP/kg) of SC rAAV/CMV.scFv X5 vectors (both with two injection sites). Similar to that observed at the 10^{11} dose, scFv X5 levels increased quickly, reaching peak levels at week 8, and decreased gradually thereafter (Figure 2.8). On average, peak levels were similar for both vectors (11.0 and 15.5 μg/ml for SS and SC vectors respectively) (Figure 2.9), with a similar kinetic profile between SC and SS vectors. These data suggest that at these extremely high dose levels, advantages gained by SC conformation are partially lost as the cells are able to produce scFv X5 as a result of strand annealing, circular episome formation or a combination of both processes. This also clearly documents that scFv constructs delivered using the above platform are unable to achieve potentially therapeutic levels. However, the secreted X5 levels did increase as a function of dose in a moderately linear fashion. For SS rAAV1/CMV.scFv X5, the vector dose increased 47-fold from 1.3x10^{11} DRP/mouse to 6.1x10^{12} DRP/mouse, while the scFv X5 serum levels increased 31-fold from 0.35 μg/ml to 11 μg/ml. For the SC vector, vector dose increased 8.5-fold from 1.3x10^{11}/mouse to 1.1x10^{12} DRP/mouse, while the scFv X5 yield increased approximately 4-fold. It is difficult to reconcile the failure to see dose saturation at these extremely high viral inputs in such a limited volume (50 μl). One possible explanation is that a portion of the vector (particularly at high doses) escaped into the systemic circulation and transduced hepatocytes or other target cells which contributed to the observed X5 levels.
2.3.3 Examination of *in vivo* scFv X5 secretion efficiency

To determine whether efficient *in vivo* secretion was occurring from the injected muscle, we sacrificed a total of 18 injected mice 16 weeks after vector administration and harvested the injected and control muscle tissues. Total soluble muscle proteins were extracted from frozen muscle tissue and the total protein concentration was measured using a BCA protein assay kit. The scFv X5 concentration in clarified muscle homogenates was measured by the gp120 binding ELISA. The total amount of scFv X5 protein in the muscle was calculated by taking the protein concentration and multiplying by the extraction buffer volume (Table 2.1). Assuming a blood volume of 2 ml, the secretion efficiency from muscle into the blood was 39±14% in average (total protein in serum/(total protein in serum + total protein in muscle), suggesting that the scFv X5 protein had been adequately secreted from the muscle into the blood circulation.

2.3.4 Long-term low-level expression of biological active scFv X5 in NHPs

Based on the above mouse data, we performed a NHP study utilizing the SC rAAV1 vector containing the SL1 leader sequence. We asked whether dosing levels used in rodents translates to the non-human primate. We reasoned that since AAV is a primate virus, the possibility exists that it will demonstrate improved transduction in the primate host at a similar dose (5x10¹² DRP/kg) (Manno, Chew et al. 2003). As a pilot experiment, two rhesus macaques (RQ6498-M1 and RQ6517-M2) were pre-screened for the absence of detectable anti-AAV1 binding antibodies using an ELISA format. Both animals were at baseline at a 1:100 serum dilution (the lowest dilution assayed), which was used as a surrogate marker for lack of natural infection. The animals received intramuscular injections into both quadriceps of 3.5x10¹³ DRP of SC rAAV1/CMV.scFv X5 vector (4 - 6x10¹² DRP/kg). Both animals displayed peak serum concentrations at week 4 (3 - 5 µg/ml), which dropped off to a steady-state plateau of ~ 0.4 µg/ml (Figure 2.11) for over one
Significantly, both animals’ sera, at early time points, possessed robust in vitro neutralization activity (titers ranged between 1:240 – 1:1400) against a CD4 independent HIV-1 isolate (NL-ADAr). This finding was consistent with the CD4i binding properties of scFv X5 (Figure 2.11). Interestingly, unlike monkey M1, monkey M2 demonstrated another burst of neutralization activity between weeks 6 to 16 with a peak value at week 10. The expression levels in both animals closely paralleled levels observed in the rodent studies with scFv X5. The peak levels in Rag-1 mice were approximately 3.5 μg/ml on average while the peak levels for the two monkeys were between 3 and 5 μg/ml (Figure 2.11). Interestingly, the mice and monkeys both reached peak levels around 4 - 8 weeks that then gradually decreased. The steady state concentration of scFv X5 was ~ 0.4 μg/ml in the two primates, while in mice it dropped less than 1.0 μg/ml over 16 weeks (Figure 2.8).

Passive immunization studies revealed that NAb concentrations in the range of 50 to 200 μg/ml are protective. While the levels observed in these animals was significantly below that believed to provide protection, the small size of scFvs allows for rapid penetration of tissues (Yokota, Milenic et al. 1992), and possibly the local concentration needed for scFv X5 to block HIV-1 infection might be lower than that determined by passive immunization. The two injected animals, along with another four naïve controls, were challenged by an R5-specific SHIVSF162P3 virus at a dose of 50 MID50 through the rectum one year post-vaccination. After one month post-challenge, no protection against the SHIV virus was achieved based on bDNA viral load data (Table 2.2). Statistically, there was no significant difference between vaccinated (monkey M1 and M2) and control groups (p > 0.05). One control animal (RQ6623) in the control group did not reach detectable viremia until week 24 post challenge, which suggested that the challenge dose of
the SHIV might need to be increased. Therefore, this low concentration of circulating scFv X5 was not effective against a virulent SHIV challenge.

2.3.5 Immune responses against transgene and rAAV1 vector

2.3.5.1 Humoral immune responses against scFv X5 and rAAV1 vector

In vivo studies with hF.IX in animal models (mice and dogs) demonstrated that inhibitory antibodies against hF.IX were formed over a wide range of vector doses administrated into muscle tissue. The inhibitory antibodies diminished systemic hF.IX levels (Wang, Cao et al. 2005). To find out whether expressed scFv X5 had stimulated the host to produce anti-X5 antibodies, western blot experiments were performed on serum samples of monkeys M1 and M2. As the Figure 2.12 shows, monkey M2 produced a detectable anti-X5 antibody response. The immune response was detected at week 6, peaked at week 8, and gradually declined with time. The fact that the scFv X5 concentration did not decrease in monkey M2 with the occurrence of the anti-X5 antibodies suggests that these anti-X5 antibodies did not diminish scFv X5 binding to gp120 in vitro. On the contrary, a corresponding neutralizing peak occurred in monkey M2 during the same period (Figure 2.11), which suggested that anti-X5 antibodies might help the host neutralize HIV-1, possibly through anti-anti-X5 antibodies (see discussion for details). As expected, a humoral immune response against the vector was detected in both of the animals by ELISA. As the Figure 2.13 shows, anti-AAV1 antibodies were produced as early as week 2 and were maintained.

2.3.5.2 Cellular immune response against rAAV1 vector

Since scFv X5 levels declined over time, the possibility that a cellular immune response was responsible for the loss was explored. Specifically, we asked whether the host produced an anti-
capsid cellular immune response as seen in rAAV mediated F.IX gene transfer to liver (Manno, Pierce et al. 2006; Mingozzi and High 2007; Mingozzi, Maus et al. 2007). I measured anti-capsid responses using a monkey IFN-γ ELISpot. Frozen PBMCs, isolated using Ficoll-Paque density gradients, were incubated with anti-IFN-γ capture antibody (MD-1) coated 96-well plates at a cell density of 4x10^5 cells/well with one of 3 AAV1 peptide pools at 0.4 μg/well. The peptide pools spanned the entire AAV1 VP1 protein (18 mers overlapping by 11). ConA served as a positive control. To exclude non-specific stimulation, a pool of eGFP peptides was used as a negative control. As the Figure 2.14 shows, no AAV1-specific cellular immune responses were detected. The positive control wells had more than 1,000 SFCs/1x10^6 PBMCs. As expected, no eGFP-specific cellular immune response was observed. We note that we did not measure anti-transgene cellular responses to the human scFv X5 protein. This remains a possible target for immune clearance.

2.4 Discussion

2.4.1 Comparison of expression levels between scFv X5 and IgG1b12

The goal of my studies was to increase the serum concentration of anti-HIV-1 neutralizing antibodies in vivo. I demonstrated that an optimized leader peptide (at least in cell culture) and a SC vector did result in increased mouse serum concentrations. When tested in a large animal model, stable scFv X5 levels were maintained over one year and neutralization activity was imparted. The data showed that the expression levels are scalable from rodents to the rhesus macaque. As far as we know, this is the first time anti-HIV-1 NAb activity was achieved in NHPs using antibody gene transfer. Our approach to increase serum antibody concentrations was based on four changes compared to the initial IgG1b12 work: 1) use of a scFv antibody; 2) use of a synthetic leader peptide; 3) use of a SC vector; and 4) use of serotype 1. In the first case, we
exploited the ability to use a cloned antibody fragment with predetermined specificity and better binding capacity than its corresponding whole IgG parent protein (Moulard, Phogat et al. 2002). This approach, combined with phage-display technology, has been used widely in recent years to generate many monoclonal antibodies with useful properties (Binley, Wrin et al. 2004). Importantly, scFvs have better tissue penetration and the small size can fit into a SC vector. In addition, scFv antibody genes only require one promoter and do not require heavy and light chain association. Secondly, synthetic leader peptides provide efficient secretion in vitro and likely in vivo (Table 2.1). These experiments support further use of these leaders to maximize protein secretion (Barash, Wang et al. 2002). Thirdly, the SC vectors have been reported to provide 10 - 100 fold greater gene expression than SS vectors. This coupled with the ability of rAAV1 vectors to provide increased gene transfer to muscle by an order of magnitude over rAAV2 vectors (Xiao, Chirmule et al. 1999; Chao, Liu et al. 2000; Riviere, Danos et al. 2006) should have led to a dramatic increase in X5 levels. X5 binds with high (nM) affinity to a variety of Envs including those from primary isolate SF162 (challenge virus env) (Moulard, Phogat et al. 2002). scFv X5 can inhibit infection of PBMCs by strain SF162 with a 50% inhibitory concentration at 5.3 μg/ml (Labrijn, Poignard et al. 2003) and has an estimated IC90 of around 10 μg/ml. SHIVSF162P3, a chimeric simian-human immunodeficiency virus passaged in vivo three times (Harouse, Gettie et al. 2001), has the Env from primary isolate SF162 and is efficiently neutralized by scFv X5. Passive immunization studies predict protection at a concentration of 100 times of IC90, which is about 1000 μg/ml for this particular antibody and the challenge virus pair (Parren, Marx et al. 2001). Clearly, the levels obtained were far below that predicted to provide any benefit.
2.4.2 Antibody half-life may be a key variable for the low expression

The question then becomes why were NAb levels not increased compared to the original IgG1b12 experiments in spite of all the improvements detailed above? It now appears that the half-life of the expressed protein may be a key variable to obtain high-level antibody expression. One presumed advantage of using scFv antibodies was that their smaller size might allow better secretion efficiency and solid tissue penetration. Local concentration of NAb is very important for blocking viral infections, particularly mucosal HIV-1 infection. One limitation of small molecules (< 40 kD) is that they are cleared more quickly through the circulatory system by glomerular filtration (Colcher, Bird et al. 1990; Milenic, Yokota et al. 1991). Moreover, pharmacokinetic analysis provides a plausible explanation for the limitation in scFv X5 levels even at high vector dosages. Making the assumption that the secreted X5 protein models a constant drug infusion paradigm, the drug (scFv X5) concentration will then eventually reach a steady state level, where production is in equilibrium with degradation. The mean steady state concentration of the drug is described by the following equation (Ritschel W.A. 2004):

\[
C_{ss} = \frac{D \cdot f}{(V_d \cdot K_{el} \cdot \tau)} [\mu g/ml]
\]  

(1)

Here in the equation, \(C_{ss}\) is the mean steady state concentration, \(V_d\) is the distribution volume, \(D\) is the drug dose administered in [\(\mu g\)] (scFv X5), \(f\) is the fraction of dose absorbed, and \(\tau\) is the dosing interval expressed in hours. \(K_{el}\) is the drug elimination constant, which is directly related to the drug half-life \((T_{1/2})\) by the following equation:

\[
T_{1/2} = \frac{0.693}{K_{el}}
\]  

(2)
Therefore, $K_{el}$ can be calculated by $T_{1/2}$, and can be exchanged to the above equation (1). Since
the scFv X5 antibody was produced in a continuous manner, we can use the above equation with
a small modification. We can assume $f = 0.39$ due to the secretion efficiency. We can also define $P = D/\tau$ that is the production rate in $\mu$g/day or $\mu$g/h. $P$ will be a constant for a certain regimen
with a fixed dose of the virus in our case. Therefore, the equation becomes the following:

$$
0.39 \cdot P
$$

$$
C_{ss} = \frac{0.563 \cdot T_{1/2} \cdot P}{0.693 \cdot V_d}
$$

For a given animal, $V_d$ is a fixed number. Therefore, with the same production rate, the steady
state concentration is directly related to the antibody’s half-life - the longer the half-life, the
higher the steady state antibody concentration. Considering scFv X5 has a half-life of minutes to
hours, while IgG1b12 has a half-life of ~ 21 days, a 100 to 1000-fold difference in the steady
state concentrations at the same production rate is possible. This huge difference in the calculated $C_{ss}$ may explain why we need higher viral doses for scFv X5 to achieve similar or better yields.

In retrospect, IgG1b12 reached a steady state concentration of 4.4 $\mu$g/ml at a dose of $5 \times 10^{11}$ DRP.
With a similar viral dose, the steady state level of scFv X5 is no more than 4.5 $\mu$g/ml (Figure 2.10). We can assume that the production rate of scFv X5 is about 200 fold higher than IgG1b12 since we used SC rAAV1 instead of SS rAAV2. If we plug all these data into the above equation
and assume a half-life of 21 days for IgG1b12, the half-life of scFv X5 is only 2.5 hours. Thus,
new strategies to improve the half-life of scFv X5, while still maintaining its biological activities,
are warranted. A possible solution to this problem may be a fusion of scFv X5 with a stable partner. However, steric hindrance may be problematic due to the epitope recognition site for this molecule.
2.4.3 Dose saturation

In this study, we tried to define an upper dose saturation limit in muscle tissue using Rag-1 mice. Surprisingly, we found out that doses of up to $5 \times 10^{13}$ DRP/kg to $3 \times 10^{14}$ DRP/kg of AAV1 by direct intramuscular injection gave fairly linear increases in X5 serum levels. However, as Figure 2.8 and Figure 2.10 shows, the scFv X5 concentrations reached peak levels and dropped significantly afterwards. This trend was not observed for medium doses ($\sim 5 \times 10^{12}$ DRP/kg) of the SS vector (Figure 2.8 and 2.9) and may suggest that transient strand-annealing, ER stress responses, or additional sites are contributing to the transient peak in expression observed. While these doses did not appear to saturate the injection site, they are likely beyond a clinically relevant dosing schedule. Typically, a child is 30 kg and an adult is 70 kg, which translates to a dose of more than $10^{14}$ DRP/person – something not achievable for a few patients let alone for millions of vaccinees. Furthermore, we did not achieve stable expression in a therapeutic range even with these high doses. Therefore, other strategies to increase transgene expression are warranted.

2.4.4 Immune responses against vector and transgene

A humoral immune response against scFv X5 was produced in monkey M2, while this was not observed in animal M1. Generally, human immunoglobulins share a high degree of homology with their primate counterparts. For example, DNA sequence similarity between macaque germline V-regions IGHV1, IGHV3, IGHV4, and IGHV7 and their closest matching human counterparts are 88.6, 87, 90 and 92.7% respectively (Andris, Miller et al. 1997; Bible, Howard et al. 2003). The lambda light-chain germline sequences for subgroups IGLV1, IGLV2, IGLV3, IGLV4, and IGLV5 exhibit 89.3, 88.6, 89.0, 94.7, and 87.1% homology to their human counterparts, respectively (Howard, Bible et al. 2005). In addition, new epitopes in scFv X5 may be generated from the cloning process: 1) the linker region between $V_H$ and $V_L$; 2) new tertiary
interaction between $V_h$ and $V_l$. Therefore, human scFv X5 could potentially be immunogenic, which could affect its persistence and in vivo expression levels. Interestingly, the anti-X5 antibodies did not influence the scFv X5 concentration as compared with the expression levels of monkey M1 at the same time. However, M2 had a second neutralization peak that correlated to the expression of these anti-X5 antibodies. Conceivably this secondary burst of neutralization activity might be explained by the ability of idiotypic immune responses that are layered upon each other and have the capacity to mirror a NAb response (network hypothesis, see Figure 1.8 and introduction Section 1.3.3 in Chapter 1). For example, since monkey M2 had high levels of anti-X5 antibodies (called Ab2) during this period (Figure 2.12), these idiotopes might mimic epitopes on the envelope glycoprotein to stimulate the monkey to produce anti-idiotypic antibodies (i.e., Ab3). The Ab3 might neutralize NL-ADArs. The fact that the second neutralization peak for monkey M2 is around week 10 (Figure 2.11, M2 (Nt)) while the anti-X5 antibody level peaked at week 8 (Figure 2.12) is consistent with this hypothesis. Such a response, if consistently elicited, may be of value. Such strategies are being attempted using designer gp120-like epitopes called mimotopes to try and elicit such NAbs (Humbert, Rasmussen et al. 2008).

As expected, a humoral immune response against the vector was produced and presents a major obstacle to the re-administration of this vector for boosting applications. Although rAAV does not have any viral protein coding genes, the capsid proteins, which were brought in when the virus infected muscle cells, could stimulate host immune responses and clear the antibody expressing cells (Nathwani, 2002; Manno, 2006; Mingozzi, 2007). Recently, IFN-γ ELISPOT assay has been employed to measure antigen specific T-cell responses using overlapping peptide pools (Tobery and Caulfield 2004; Wang, Sin et al. 2004; Zhang, Huang et al. 2006). However, we did not find
any positive cellular responses when we applied the Cap1 peptide pools during several early time points measured (Figure 2.14). Our ELISpot sensitivity might be low since we used unstimulated frozen PBMCs (Mingozzi, 2007). Therefore, we still cannot exclude the possibility that a low level cellular immune response against the vector decreased transgene expression.

In conclusion, we have demonstrated that scFv X5 can be successfully expressed in mice and in NHPs in a scalable manner through rAAV1 mediated gene transfer. The SL1 leader sequence facilitated efficient secretion. The SC vector was about 3 - 14 fold better than the SS rAAV1 vector at the same vector dose. We also showed that no dose saturation was found at medium (5x10^{12} DRP/kg) as well as high doses (5x10^{13} – 3x10^{14} DRP/kg) of rAAV1/CMV.scFv X5 in Rag-1 mice. Furthermore, low-level expression of scFv X5 over one year in rhesus macaques did not achieve protection against a virulent intra-rectal SHIV challenge. New strategies are needed to increase the NAb concentrations in vivo. Increasing scFv half-life appears to be a logical target for future studies.
Table 2.1: *In vivo* scFv X5 secretion efficiency

<table>
<thead>
<tr>
<th>Vector</th>
<th>Animal #</th>
<th>Dose (DRP)</th>
<th>Inj. Vol</th>
<th>X5 Level (μg)</th>
<th>Total in Blood (2 ml)</th>
<th>Total in Muscle</th>
<th>% Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV1/eGFP</td>
<td>A1</td>
<td>2.2x10⁹</td>
<td>50 μl</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>2.2x10⁹</td>
<td>50 μl</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0%</td>
</tr>
<tr>
<td>SC rAAV1/X5</td>
<td>D1</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>1.88</td>
<td>9.76</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>3.60</td>
<td>4.13</td>
<td>47%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>3.80</td>
<td>12.46</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D4</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>1.54</td>
<td>9.34</td>
<td>14%</td>
<td></td>
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<tr>
<td>SC rAAV1/X5</td>
<td>E1</td>
<td>1.3x10¹¹</td>
<td>200 μl</td>
<td>0.60</td>
<td>0.44</td>
<td>57%</td>
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<tr>
<td></td>
<td>E2</td>
<td>1.3x10¹¹</td>
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<td>0.68</td>
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<td>47%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>1.3x10¹¹</td>
<td>200 μl</td>
<td>0.92</td>
<td>2.92</td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>1.3x10¹¹</td>
<td>200 μl</td>
<td>0.60</td>
<td>0.44</td>
<td>57%</td>
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</tr>
<tr>
<td>SS rAAV1/X5 4 injections</td>
<td>F1</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>0.84</td>
<td>0.64</td>
<td>57%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>0.73</td>
<td>0.93</td>
<td>44%</td>
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<tr>
<td></td>
<td>F3</td>
<td>1.3x10¹¹</td>
<td>100 μl</td>
<td>1.40</td>
<td>&lt; 0.12</td>
<td>&gt;92%</td>
<td></td>
</tr>
<tr>
<td>SS rAAV1/X5 2 injections</td>
<td>G1</td>
<td>1.3x10¹¹</td>
<td>50 μl</td>
<td>0.49</td>
<td>0.84</td>
<td>37%</td>
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<tr>
<td></td>
<td>G2</td>
<td>1.3x10¹¹</td>
<td>50 μl</td>
<td>0.63</td>
<td>1.10</td>
<td>36%</td>
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<tr>
<td></td>
<td>G3</td>
<td>1.3x10¹¹</td>
<td>50 μl</td>
<td>1.10</td>
<td>1.10</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>SS rAAV1/X5 2 injections</td>
<td>H1</td>
<td>6.1x10¹²</td>
<td>100 μl</td>
<td>11.2</td>
<td>10.3</td>
<td>52%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H2</td>
<td>6.1x10¹²</td>
<td>100 μl</td>
<td>11.6</td>
<td>15.7</td>
<td>43%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H3</td>
<td>6.1x10¹²</td>
<td>100 μl</td>
<td>11.7</td>
<td>18.4</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total in Blood (2 ml)</td>
<td>Total in Muscle</td>
<td>% Secreted</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39 ± 14%</td>
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</tr>
</tbody>
</table>

Note: no scFv X5 was detected in the muscle sample in mouse F3. It is most likely due to sampling error. Therefore, this data was not included in the average.
Table 2.2: Viral loads before and after challenge (bDNA, $\log_{10}$ copies/ml)

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Animal ID</th>
<th>WK00</th>
<th>WK02</th>
<th>WK04</th>
<th>WK12</th>
<th>WK24</th>
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<tr>
<td></td>
<td></td>
<td>12/14/05</td>
<td>12/29/05</td>
<td>1/11/06</td>
<td>3/08/06</td>
<td>5/31/06</td>
</tr>
<tr>
<td>X5 Vaccine</td>
<td>RQ6498(M1)</td>
<td>&lt;1.5</td>
<td>5.0</td>
<td>3.6</td>
<td>3.4</td>
<td>&lt;1.2</td>
</tr>
<tr>
<td></td>
<td>RQ6517(M2)</td>
<td>&lt;1.5</td>
<td>6.9</td>
<td>5.6</td>
<td>3.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Control</td>
<td>RQ6623</td>
<td>&lt;1.5</td>
<td>&lt;1.5</td>
<td>&lt;1.5</td>
<td>&lt;2.1</td>
<td>2.2</td>
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<td>RQ6628</td>
<td>&lt;1.5</td>
<td>7.5</td>
<td>5.5</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
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<td>7.7</td>
<td>4.1</td>
<td>3.2</td>
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<td>7.3</td>
<td>5.2</td>
<td>2.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Figure 2.1: Branched DNA specimen processing (Tsongalis 2006). A: Stage of RNA release and probe hybridization. Virus is collected from animal’s plasma and the viral RNA is exposed by proteinase K digestion. Label extenders and capture extenders were then hybridized with high stringency to the virus RNA. Capture extenders were designed to hybridize to the viral RNA and capture probes, which were immobilized on the micro well plate. Label extenders were designed to hybridize to the regions next to the capture extenders and to provide sequences to hybridize to a preamplifier oligonucleotide. B: Stage of bDNA signal amplification. The preamplifier was used to hybridize to multiple amplifier molecules that created a bDNA structure. For chemiluminescent detection, alkaline-phosphatase (AP)-labeled oligonucleotide probes bound to the bDNA molecule by hybridization. The chemiluminescent product of the AP reaction is the bDNA signal. Permission to use the figure was granted.
Figure 2.2: HIV-1 neutralization assay using TZM-bl cells (Wei, Decker et al. 2002).
A: Diagram of lentivirus vector used to transduce TZM-bl cells (LTR: long terminal repeat; SD: splice donor; Ψ: RNA packaging signal; RRE: rev-responsive element; IRES: internal ribosomal entry site). The β-gal and luciferase genes were both introduced into TZM-bl cells and cell line expresses high-levels of CD4, CCR5 in the absence of constitutive of β-gal and luciferase. B: Example of preparation of pseudotyped progeny virions. Full-length env genes were cloned into the pCDNA3.1 eukaryotic expression plasmid. To make pseudotyped virions, individual pCDNA3.1-env clones were co-transfected with plasmid pSG3 containing a HIV-1 backbone without env gene into 293T cells. C: Pseudotyped virus produced from 293T cells were used to infect TZM-bl cells in either the absence (control) or the presence of various concentration of NAb or vaccinated animal serum. The results were expressed as TCID$_{50}$, which is the value of the serum dilution at which relative luminescence units were reduced by 50% compared to virus control wells without NAb or vaccinated monkey serum. Permission to use the figure from the American Society of Microbiology was granted.
Figure 2.3: Illustration of pAAV/CMV.scFv X5 constructs with different leader peptides. The 2.0 kb expression vector cassette contains the IE CMV promoter/enhancer (760 bp) and a 240 bp SV40 polyA tail. The human scFv X5 contains the variable light IgG region (360 bp) linked to the variable heavy (360 bp) IgG domain. The linker is a (4GS)_3 motif. Different leader peptides SL1 (A), SL2 (B), and NL (C) were inserted before scFv X5 gene.
Figure 2.4: Western blot detection of scFv X5 following plasmid DNA transfection of HeLa cells. HeLa cells (3x10^5) were transfected with 2 μg of plasmid DNA in a 6-well format. Synthetic leader-CMV-X5 expression constructs (SL1 and SL2) were generated by overlapping PCR, using the native leader CMV.scFv X5 plasmid (NL) as the PCR template. Cell supernatant (Sup) was diluted 1:2 (20 μl loaded) and the cell pellet (CP) was resuspended in 200 μl SDS loading buffer and 20 μl loaded onto a 10% SDS gel. Immunodetection was achieved by incubation with HRP conjugated Protein-L (does not cross react with bovine IgG) and the ECL Detection System.
Figure 2.5: ELISA assay of cell supernatant of transfected HeLa and C2C12. OD450nm values from the ELISA assay (see materials and methods) are plotted versus the samples tested. Transfection efficiency was normalized by measuring β-gal activity in the cell lysate (the cell was co-transfected with the same amount of pAAV/CMV.β-gal). For the purpose of comparison, cell supernatants from SL1 and SL2 based vectors transfected into HeLa cells were diluted 100 fold. The other cell supernatants were tested directly without dilution.
Figure 2.6: Cloning schematic of SC rAAV1/CMV.scFv X5/Cap1-Rep2-NeoTK and virus production. The 2.0 kb scFv X5 expression cassette was cloned into SC base vector pHpa7 using Hpa I and Xba I restriction sites. The fragment containing cap1, rep2, and NeoTK (i.e., C1-R2-SV40-NeoTK) was inserted into the new SC vector B54 at the unique Swa I site. The resulting producer triple-play plasmid was then used to transfec low passage HeLa cells for cell line screening. The best cell line (DRP/cell) was used for virus production.
**Figure 2.7: Purity of SC rAAV1/CMV.scFv X5.** rAAV1 (SC rAAV1/CMV.scFv X5) was purified from a stable HeLa producer cell line (L21021) following Ad5 infection (moi = 40). A crude cell lysate was generated by 4 freeze/thaw cycles and Benzonase digestion (35 U/ml). The lysate was purified by iodixanol step gradient and diluted 10-fold prior to application on an anion exchange column. Virus was eluted using a NaCl gradient. 20 μl of pooled peak fractions were run on 10% SDS-PAGE gel and protein visualized by SYPRO Orange staining. VP1 (83 kD), VP2 (73 kD), and VP3 (62 kD) are labeled by black arrow heads. Purified rAAV2 (rAAV2/CMV.β-gal) was used as a molecular weight reference.
Figure 2.8: scFv X5 concentrations in Rag-1 mice injected with SC and SS vectors. Two groups of Rag-1 mice (n = 3) were injected with the same amount of SC or SS rAAV1.CMV.scFv X5 virus (1.3x10^{11} DRP in 50 μl volume in PBS buffer). Control mice were injected with rAAV1/CMV.eGFP at the same dose. The mice were bled biweekly. The serum scFv X5 concentration was quantitated by gp120 ELISA using a known scFv X5 standard kindly provided by Dr. Dennis Burton.
Figure 2.9: Effect of multiple injections. Three groups of Rag-1 mice (n = 3) were injected with the same amount of SC and SS rAAV1/CMV.scFv X5 virus (1.1x10^{11} DRP in 50 μl volume in PBS buffer). The virus was divided into one, two, or four doses for single (SS1), two (SS2), or four (SS4) injections, respectively. Control group was injected with the same amount of rAAV1/CMV.eGFP at one dose for single injection. The mice were bled biweekly. The serum scFv X5 concentration was quantitated by gp120 ELISA using a known scFv X5 standard.
Figure 2.10: scFv X5 concentrations in Rag-1 mice injected with high dose SC and SS vectors. Two groups of Rag-1 mice (n = 3) were injected with different amounts of SC and SS rAAV1/CMV.scFv X5 virus (1.1x10^{12} DRP in 50 μl volume in PBS buffer for SC vector, 6.1x10^{12} DRP for SS vector). Control group was injected with 1.1x10^{12} DRP of SS rAAV1/CMV.eGFP. The mice were bled biweekly. The serum scFv X5 concentration was quantitated by gp120 ELISA using a known scFv X5 standard.
Figure 2.11: Rhesus macaque serum scFv X5 concentrations and neutralization activity post vaccination. Two rhesus macaques were injected with SC rAAV1/CMV.scFv X5 and sera were drawn biweekly. The serum scFv X5 concentration was quantitated by gp120 ELISA using a known scFv X5 standard. Neutralization activity was measured by using a luciferase reporter gene assay in TZM-BL cells (details, please refer to the materials and methods Section). A: scFv X5 concentrations and neutralization activity against NL-ADArS. B: scFv X5 neutralization activity. Values are the serum dilution at which relative luminescence units (RLU) were reduced 50% compared to virus control wells (no test sample).
Figure 2.12: Western strip blot analysis of anti-idiotypic antibodies against scFv X5 in monkey serum. 1.0 μg of purified scFv X5 was run on a 10% SDS-PAGE. After the protein was transferred to a Hybond-p membrane, the strip was hybridized with monkey serum from each individual week at 1:100 dilution. After the secondary antibody (rabbit anti-monkey IgG HRP conjugate) was incubated with the strip at 1:10,000 dilution at room temperature for 30 min, the strips were visualized using the ECL kit (details, please refer to the materials and methods section).
Figure 2.13: Humoral immune response against rAAV1 detected by ELISA. OD450nm values from the ELISA assay are plotted versus the different week serum samples from monkey M1 and M2. W0 was the pre-bleed before the vector injection. All the sera were diluted at 1: 100 in PBST.
Figure 2.14: IFN-γ ELISpot assay on the PBMCs from rAAV1/CMV.scFv X5 injected monkeys using VP1 peptide pools. The experiment was performed in a 96-well plate. The top line indicates which peptide pools (P1, P2, P3, or Pe) used for the cells in that column. P1, P2, P3 were three peptide pools of AAV1 VP1. Con A was used in the columns labeled as C+, which served as positive controls. Pe was the peptide pool of eGFP, which was used as a negative peptide control for the cells in the columns. No peptide pool was used in the columns labeled as C-, which served as negative cell-only controls. Each side of the plate labels the PBMCs used for that half row from different monkeys and different weeks. Each cell samples were duplicated. M1wo and M2wo were the PBMCs from monkey M1 and M2 before vaccination; the other cells were from monkey M1 or M2 at week 8, 10, and 12 (as labeled in their name). For more details, please see materials and methods.
CHAPTER 3

OPTIMIZATION OF SCFV ANTIBODY GENE
DELIVERY AND EXPRESSION

3.1 Introduction

A novel HIV-1 vaccination approach was detailed in Chapter 2. The vaccine vector encoded a rare, broadly neutralizing scFv antibody termed X5 and it was successfully expressed in a mouse model. Based on these promising murine experiments, we scaled the vector dose and vaccinated two rhesus monkeys. Similar to the mouse studies, we successfully expressed scFv X5 in this larger animal model. Unfortunately, the expression levels were too low to prevent infection following a virulent SHIV challenge. In this chapter, we explore additional strategies to increase transgene serum levels in the mouse and monkey models. These strategies include: 1) using a fusion partner to increase scFv half-life; 2) using a new scFv antibody which is even more potent and broader than scFv X5; 3) testing another strong promoter to drive the transgene expression; 4) using the woodchuck hepatitis post-transcriptional regulatory element (WPRE) to increase transgene translation; and 5) trying an additional AAV serotype besides AAV1.
3.1.1 Genetic fusion to increase antibody half-life

Pharmacokinetic analyses showed that scFv antibodies have much more rapid plasma and whole-body clearance than native antibodies (Milenic, Yokota et al. 1991; Yokota, Milenic et al. 1992). Several technologies have been developed for extending the half-life of therapeutic proteins including: 1) use of liposomes to slow the delivery of the protein into the blood stream (Longman, Tardi et al. 1995); 2) chemical modifications of the protein by linking it to large polymers (Gonen-Wadmany, Oss-Ronen et al. 2007); and 3) genetic fusions to extend half-life (Kilpatrick, Kerner et al. 2002; Martinez, Drescher et al. 2004; Schulte 2008). Our challenge is to achieve an extended half-life of the antibody while preserving its neutralization activities. For scFv X5, we constructed two genetic fusions with the rhesus macaque alpha1-antitrypsin (A1AT) serum protein at either the N or C-terminus. We chose A1AT as a fusion partner because it is a long-lived abundant, monomeric plasma protein (~ 3.0 mg/ml) that has been used successfully for genetic fusions (Vanhove, Laflamme et al. 2003). Additionally, it has been shown that concentrations of human A1AT in mouse serum can reach 800 μg/ml after a single injection of rAAV2 into skeletal muscle (Song, Morgan et al. 1998). Unfortunately, both X5-A1AT and A1AT-X5 fusion proteins lost the ability to bind gp120 based on our ELISA data, while large amounts of fusion proteins were detected by an A1AT ELISA (Figure 3.1). The likely explanation was that the increased size and globular structure of the A1AT fusion partner contributed to steric inhibition at the CD4i binding site. This is not surprising since X5 is known to be sterically constrained (Moulard, Phogat et al. 2002). Therefore, we chose to explore another scFv moiety that targets a different region at the viral envelope and may not be as susceptible to steric constraints imparted by the fusion partner. Accordingly, we identified a second scFv termed 3B3 (scFv of IgG1b12) that was isolated by Burton and colleagues (Burton, Pyati et al. 1994). 3B3 was affinity matured by Hammer and colleagues using site directed mutagenesis (McHugh,
Hu et al. 2002). It is a high-affinity anti-gp120 antibody that neutralizes a wide range of primary and laboratory isolates of HIV-1, and recognizes the same epitope as the parental antibody IgG1b12 (CD4BS). Both the potency and breadth of neutralization activity of the 3B3 were improved over IgG1b12 (Barbas, Hu et al. 1994; McHugh, Hu et al. 2002).

While working with A1AT, we learned that IgG constant regions can also be used as fusion partners. The advantage of using the constant region of IgG is apparent. IgG fusions not only increase half-life but also imparts the ability to dimerize, which allows the scFv antibodies to have properties similar to those of conventional antibodies (e.g., increased affinity and effector functions). Therefore, we decided to evaluate the constant IgG1 region (hinge-C2-C3) in the context of the 3B3 scFv fragment (Afanasieva, Wittmer et al. 2003). The use of the IgG1 hinge may allow in vivo dimerization with increased antibody avidity (Nielsen, Adams et al. 2000; Bera, Williams-Gould et al. 2001).

3.1.2 Enhanced in vivo antibody production using transcriptional and translational regulatory elements

In addition to translational fusions, we wanted to further evaluate transcriptional and post-transcriptional methods to increase serum transgene expression levels. For the transcriptional elements, the promoter plays a critical role in directing the level of transgene expression. Therefore, optimizing the promoter is a straightforward way to improve scFv in vivo expression.

In addition to the ubiquitous cytomegalovirus (CMV) promoter used in our initial studies, a CMV-chicken beta-actin hybrid (CAG) promoter was chosen for comparison (Okabe, Ikawa et al. 1997; Song, Morgan et al. 1998; Xu, Mizuguchi et al. 2001). The CAG promoter is composed of the human cytomegalovirus immediate-early enhancer, a modified chicken beta-actin transcription start site, and a rabbit beta-globin intron (Niwa, Yamamura et al. 1991). Transgenic
mice with an integrated eGFP gene driven by the CAG promoter showed eGFP expression in all tissues except erythrocytes and hair (Okabe, Ikawa et al. 1997). This experiment suggested that a CAG promoter-bearing vector would be expressed well in muscle cells in vivo. Xiao and colleagues have compared 6 promoters including the CAG and CMV promoters in transducing the inner hair cells of the cochlea in mice. They discovered that the CAG promoter directed the most robust levels of expression (Liu, Okada et al. 2007). Moreover, a study using rAAV vectors expressing human A1AT has demonstrated that the CAG promoter is nearly two-fold more efficient than the CMV promoter in mouse liver (Song, Laipis et al. 2001). Therefore, the CAG promoter was compared to the CMV promoter in our muscle-mediated scFv antibody expression studies.

For the translational regulatory elements, the WPRE is one of the best-known examples. As indicated by its name, the WPRE is derived from woodchuck hepatitis virus and contains three independent post-transcriptional regulatory elements WPREα, WPREβ, WPREγ (Donello, Loeb et al. 1998). The WPRE has been studied extensively in vitro through the addition of the WPRE to retroviral vectors which increased both luciferase and eGFP protein production 5 to 8 fold (Zufferey, Donello et al. 1999). This effect is transgene and promoter independent. The addition of the WPRE to rAAV vectors increased transgene expression in stably transformed cell lines and primary human fibroblasts (Loeb, Cordier et al. 1999). Similar results were obtained by another research group using rAd vectors in vitro to target vascular smooth muscle cells from pig coronary arteries (Appleby, Kingston et al. 2003). These experiments suggest that the strategy of post-transcriptionally enhancing gene expression with the WPRE could be broadly applicable. In our study, the WPRE is positioned after the stop codon of 3B3 and 3B3hCC to assess whether it improves serum antibody levels.
3.1.3 Use of an alternative AAV serotype (rh.74)

For rAAV mediated gene delivery targeting the muscle, serotype 2 was widely used initially (Fisher, Jooss et al. 1997; Song, Laipis et al. 2001; Lewis, Chen et al. 2002). With the discovery of other AAV serotypes, AAV1 was found to direct approximately 10-fold greater levels of transgene expression compared to rAAV2 at the same particle dose in multiple species. Recently, several additional serotypes (AAV 7, 8, and 9 and related variants) have been isolated from non-human primates. AAV8, rh.10 and rh.74 have been intensively studied in part because of the wide-tissue tropism and ability to direct robust gene expression in multiple tissues. These vectors appear to also mediate transgene expression following systematic vascular delivery (Wang, Zhu et al. 2005). In this study, we assessed whether an rAAV8-like isolate, that we cloned termed rh.74 vector (called AAV8 for simplicity), could increase scFv antibody production following local intra-muscular injection compared to AAV1.

3.2 Materials and methods

3.2.1 Construction of 3B3 and a related genetic fusion

To obtain maximal gene expression, codon optimization of the 894 bp gene sequence (3B3N31HQ100Y) was performed (McHugh, Hu et al. 2002). The synthesized gene was cloned into plasmid pCRBlunt by Retrogen Inc. The SL1 leader peptide was incorporated into the synthesized 3B3 gene. To facilitate further cloning, the gene was re-amplified from the vector by PCR with the following primers: forward primer P1-3B3 5’-CTT AGC GGC CGC CAC CAT GTG GTG GCG-3’ and the reverse primer P9-3B3Rev 5’-CTT AGC GGC CGC ACT CAC TTG CGC TCC AGC-3’. Both primers have Not I sites within the 5’ overhangs. The PCR amplified fragment (918 bp) was recovered from 2% agarose gel and cloned into a pAAV/CMV.β-gal expression plasmid to generate plasmid pAAV/CMV.3B3.
To increase protein half-life, the hinge, C2 and C3 heavy chain domains (hCC) of rhesus macaque IgG1 were utilized as a fusion partner. Briefly, total RNA was extracted from naïve rhesus monkey PBMCs by using QIAGEN RNAeasy kit (Cat. #74104, Valencia, CA). The cDNA was generated by using QIAGEN Ominscript Reverse Transcription kit (Cat. #205111) with an oligo-dT primer. Once the primer was annealed with the template in the presence of a dNTP mixture and an RNase inhibitor in RT buffer, one strand of cDNA was synthesized by a RNA-dependent DNA polymerase function. The RNA template was degraded by a hybrid-dependent exoribonuclease function and second strand cDNA synthesis occurred by a DNA-dependent DNA polymerase function. After the cDNA was synthesized, a DNA fragment which included just the IgG1 hinge, C2, and C3 regions was amplified by using the following two conserved primers designed from the published human IgG1 sequence. The forward primer was hCCStart257 5’-TTC ATG AGC CCA GCA ACA CC-3’. The reverse primer was hCCEndSpeI 5’-GGC GAC TAG TCT CAT TTA CCC GGA GAC AGG-3’. The PCR amplified fragment (725 bp) was recovered and cloned into a pCRII-TOPO TA plasmid DNA vector by TOPO TA cloning (Invitrogen Corp., Cat. #K4600-40, Carlsbad, CA). Clones containing the insert were sequenced using an ABI 727 capillary electrophoresis automatic sequencer (PE Applied BioSystems) by the NCH Sequencing Core Laboratory. Clone TOPO TA/C.2 was used as the template for the construction of the genetic fusion. To facilitate the PCR overlap cloning process, the hCC fragment was PCR amplified from TOPO TA/C.2 by using the following two primers: forward primer- P3-hCC-3B3 5’-CCA CCA CAT GTT TTT ATC TCC TTG CGC TCC AGC TTG GTG CCC- 3’ and reverse primer- P4-endhCC 5’-CTT AGC GGC CGC TCA TTT ACC CGG AGA CAG GGA GAG GC- 3’. 3B3 was also re-amplified with primers P1-3B3forw and P2-3B3-hCC 5’- CCA AGC TGG AGC GCA AGG AGA TAA AAA CAT GTG GTG GTG GC-3’, which generated a 35 bp overlap with the primer P3-hCC-3B3. The two separate
fragments were then hybridized together by the PCR overlap technique and amplified with primers P1-3B3forw and P4-end hCC. The final PCR fragment (3B3hCC) contained Not I sites at both ends, which was digested and inserted into Not I digested pAAV/CMV.3B3 to replace 3B3 transgene. This cloning process generated plasmid pAAV/CMV.3B3hCC.

3.2.2 Construction of a rAAV base vector with the CAG promoter

To compare the promoter efficiencies, the CAG promoter was also evaluated. Briefly, plasmid pCAG.Epo (a gift from the NCH Vector Core), which contains the CAG promoter and human Epo gene, was digested with Nhe I and EcoRV. A 2,189 bp fragment was band-isolated and cloned between the AAV2 ITRs to generate a base vector for the CAG promoter. Clone pAAV/CAG.Epo/C.20 was selected for further use based on restriction enzyme analysis. The vector was digested with EcoRI and HindIII to remove human Epo gene, then, end dephosphorylated and blunt ligated with the 3B3 or 3B3hCC fragments, to generate pAAV/CAG.3B3 and pAAV/CAG.3B3hCC. All DNA manipulations were performed using standard techniques (Sambrook et al., 1989, Ausubel et al., 1987).

3.2.3 Cells and viruses

293K cells (a generous gift from Dr. Brian Kaspar), a sub-clone of 293 cells, were maintained in DMEM media supplemented with 10% CCS and 2.8 μg/ml Ciprofloxin.

All viruses were produced by a modified calcium phosphate plasmid DNA transfection method. Briefly, the 293K cells were seeded at a density of 5x10^6/plate in 150x25 mm tissue culture dishes. After 2 days growth, calcium phosphate mediated DNA transfection was performed. On the day of transfection, all DNA plasmid solutions were warmed up to 50°C for 1 hour. During
this time, the cell medium was replaced with cold (4°C) 10% CCS/DMEM 2 hours before transfection. Chloroquine (Sigma, Cat. #C-6628) was added to the media in the dish at a final concentration of 50 μM, 1 hour before adding the DNA mixture. To make the DNA mixture, all solutions were warmed to 37°C: distilled sterile DNase/RNase free water (Invitrogen Corp., Cat. #10977-015), 2x HEPES buffer (50 mM HEPES, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM Na₂HPO₄·7H₂O, pH 7.05, sterile filtered), and Ca²⁺ solution (2.5 M CaCl₂, sterile filtered). The Ca²⁺ solution, as well as DNA plasmids, were diluted into water at a final concentration of 300 mM in a 50 ml conical tube and mixed well. The ratio of the DNA plasmids was 5:20:20 (transgene, Ad Helper, AAV helper, respectively). A total of 45 μg of plasmid DNA in a volume of 1 ml was prepared and slowly added drop-wise to an equal volume of 2xHEPES. A master mix of the DNA-Ca²⁺ solution for 20 dishes can easily be made in a single 50 ml conical tube. After mixing, a fine precipitate of Ca²⁺-DNA mixture should form. The solution is then immediately added drop-wise to each dish (total 2 ml/dish). The medium is replaced with fresh 10% CCS/DMEM media the next morning. After another 3 days in culture, the cells were harvested. Both the cell supernatant and the pellet were saved for virus purification. Transfection efficiencies of 70 – 90% can be routinely achieved and up to 80% of the total virus can be found in the cell media (data not shown). Yields of up to 10¹³ DRP/dish were obtained with high efficiency transfection. The purification process was the same as reported in Chapter 2 except that the virus present in the media was also purified and combined with the virus from the cell pellet. The collected media was condensed first by using a tangential flow filtration system (AKTA Cross-flow, GE Healthcare Biosciences AB, Uppsala, Sweden) with a 100 kD molecular cut-off membrane.
The final virus stock was also tested for purity and quality. The level of endotoxin contamination was measured using an LAL Endotoxin Detection kit according to the manufacturer’s guidance (MP Biomedicals, Cat. #3070000). Sterility was confirmed using tryptic soy broth (TSB) and thioglycolate broth incubation. Purity was assessed by SDS-PAGE analysis. Typically, 20 μl of virus stocks were mixed with 4 μl 6xSDS loading buffer and analyzed on a 12% SDS gel (Bio-Rad, Cat. #161-1633). After electrophoresis, the gel was soaked in 7.5% acetic acid solution containing 10 μl SYPRO Orange (Bio-Rad, Cat. #1703120) for 1 hour. After several rinses with 7.5% acetic acid solution, the protein bands were visualized using a Gel logic 200 Imaging System (Kodak Molecular Imaging Systems, Cat. #GL200).

3.2.4 Animals

All experiments involving animals were approved by Nationwide Children's Research Institute Animal Care and Use Committee. The usage of Rag-1 mice and rhesus macaques were similar as described in Chapter 2 except that the four injected and four control rhesus macaques were randomly selected. The challenge virus and the experimental procedure for rhesus macaques were the same as those detailed in Chapter 2. After 42 weeks post challenge, all the animals were euthanized for vector biodistribution analysis. The injected quadriceps muscle tissues as well as the same areas in the control animals were harvested and dissected to 9 sections. The samples were stored frozen at -80°C until DNA extraction. Freshly thawed tissues (0.1 – 0.3 g) were frozen using liquid nitrogen and ground quickly into powder using a mortar and pestle. The sample was then transferred into a 15 ml conical tube and incubated for 18 hours in 3 ml of tissue digestion buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 0.5% SDS, 25 mM EDTA) supplemented with 2 mg/ml proteinase K at 56°C. Total DNA in the tissue sample was then subjected to three phenol/chloroform/isoamyl alcohol (Invitrogen, Cat. #15593-031) extractions in a 15 ml tube
using phase lock gel light (Fisher Scientific, Cat. #2302840). After a final chloroform extraction, DNA was ethanol precipitated, air dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). AAV genome copy numbers in the tissue samples were then quantified using real-time TaqMan PCR analysis (ABI 7700, PE Applied BioSystems) using a CMV promoter specific primer and probe set (Schnepp, Jensen et al. 2005).

### 3.2.5 Enzyme immunoassay

3B3 and 3B3hCC levels in the animal sera were measured using a modified ELISA protocol similar to that used for scFv X5 detection as mentioned in Chapter 2. Briefly, LAI/gp120 (Progenics pharmaceuticals, INC, Cat. #12101) was used to coat 96-well ELISA plates (100 ng/well) in PBS buffer at 4°C overnight. After removal of the coating buffer, each well was blocked with 285 μl of 3% BSA/PBST (blocking buffer) for 3 hours with constant shaking at 25°C. After 4 PBST washes, wells were incubated with 100 μl of diluted serum or an IgG1b12 standard (gift from Dr. Dennis Burton) in PBST for 1 hour at 25°C. Protein L-HRP conjugate (Pierce, Cat. #32420, Rockford, Il) was used as the secondary antibody at a 1:250 dilution. After 4 PBST washes, 100 μl of diluted Protein L in the blocking buffer was added to each well and incubated at 25°C for 2 hours. After 4 times washing in PBST again, the plate was developed by addition of 100 μl of TMB substrate (One-Step Ultra TMB-ELISA, Pierce, Cat. #34028) for 20 min. at 25°C. The reaction was stopped by adding 100 μl 1N H₂SO₄ to each well and the OD₄₅₀nm was measured using the SpectraMax M2 plate reader (Molecular Devices, Cat. #D02090). Serum antibody concentrations were calculated from the protein standard curve fitted by the SoftMax 6.2 with a linear or 4-parameter fit. Only curves with r² values of ≥ 0.99 were used for concentration calculations. The assay sensitivity was demonstrated to be 1.25 ng/ml of IgG1b12.
The heat stability of *in vitro*- and *in vivo*-produced scFv proteins was tested by a modified gp120 binding ELISA. Briefly, 20 μl aliquots of animal serum were heated on a dry block incubator for 0, 15, 30, 45, or 60 min. at either 53°C or 56°C. Purified proteins were either heated in PBS buffer or spiked in naïve animal serum at a dilution of 1:10 and heated at 56°C for the indicated times. Control samples were kept on ice. After heating, the samples were transferred immediately onto ice. Samples were diluted and applied to the wells of a gp120 coated ELISA plate to measure the binding capability of the protein. Detection of anti-AAV antibodies was performed according to the ELISA method described in Chapter 2.

### 3.2.6 Purification of the recombinant antibodies

scFv 3B3 and the genetic fusion 3B3hCC were purified based on their binding capabilities with Protein L. Briefly, 293K cells were transiently transfected with plasmid pAAV/CMV.3B3 or pAAV/CMV.3B3hCC in serum free media (MediaTech, Cat. #MT40-200-CV). The media was harvested 72 hours post-transfection and clarified by centrifugation at 2,000g for 20 min. The media was diluted 1:1 with PBS and mixed with Protein L agarose (Pierce, Cat. #20510) in a 250 ml conical centrifuge bottle (1 ml agarose beads/100 ml media supernatant). The bottle was rotated slowly overnight at 4°C. The mixture was then spun at 1,000g for 10 min. at 4°C. The supernatant was gently poured off, and the agarose beads were packed into a small column. The column was washed with 15 ml PBS and bound protein eluted (three times) with 1 ml IgG elution buffer (Pierce, Cat. #21004). The elution buffer is a stabilized primary amine-containing buffer at pH 2.8, which efficiently disrupts the antibody-antigen interaction. Immediately after elution, the protein solution (1 ml/tube) was mixed with 100 μl 1M Na₃PO₄ buffer (pH 8.0) to neutralize the pH. The purified protein fractions were combined and dialyzed against PBS buffer. After dialysis, the purified protein was stored at -80°C.
3.2.7 Western blot

Western blot analysis was performed to detect whether scFv 3B3hCC dimerizes in cell culture and in vivo when produced and secreted from mouse muscle. To reduce background signal, 3B3hCC from mouse serum was purified using ImmunoPure Immobilized Protein L Plus spin kit following the protocol provided by the manufacturer (Pierce, Cat. #20530). Briefly, 200 μl of ImmunoPure Immobilized Protein L Plus agarose beads were dispensed into a disposable Spin Cup Column. After the column was washed twice with 300 μl PBS, 200 μl of diluted serum sample (1:1 in PBS) was loaded onto the spin column and the reaction was incubated at 25°C for 30 min. with constant shaking. The spin column was then centrifuged at 1000g for 1 min. After the column was washed three times with 400 μl PBS, the protein was eluted with 400 μl of low pH IgG elution buffer into a new collection tube. The eluted 400 μl fraction was neutralized immediately with 40 μl 1M sodium phosphate buffer (pH 8.0). The same procedure was used for naïve control mouse serum (negative control) sample for the western blot. To determine whether 3B3hCC forms a dimer in vitro and in vivo, samples of cell culture media and the column purified material from mouse sera were run on a 7.5% SDS-polyacrylamide gel with or without 5% β-mercaptoethanol. Ten μl Magic Marker XP (Invitrogen, Cat. #LC5602) was used as a molecular marker. The proteins were then transferred to a Hybond-P membrane and the membrane was blocked with 5% nonfat dry milk in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, 0.1% Tween 20). Immunodetection was achieved by incubating the membrane with HRP conjugated Protein L at a 1:5,000 dilution in blocking buffer and using the ECL Plus Western Blotting Detection System (Amersham Pharmacia, Cat. #RPN2132).
To detect humoral immune response against the transgene product, a strip western blot was performed. A similar procedure as described in Chapter 2 Section 2.2.9 was employed. Purified 3B3, instead of scFv X5, was used as the target antigen and transferred to the membrane.

3.2.8 HIV neutralization assay

Neutralization was measured in a modified luciferase reporter gene assay using different pseudovirions or primary isolates. These experiments were performed in Dr. David Montefiori’s laboratory as described in Chapter 2 Section 2.2.8 both with and without the heat treatment.

3.2.9 Immunohistochemical detection of 3B3hCC

Immunohistochemical detection of 3B3hCC in mouse muscle tissue was performed following previously published procedures (Lewis, Chen et al. 2002). Since human and rhesus IgG1 share very high homology, we used the same primary and secondary antibodies, as in the referenced publication, to detect the expression of 3B3hCC, which contains a rhesus IgG1 heavy-chain Fc domain. Serial mouse muscle paraffin sectioned (4 μm) were deparaffinized (Americlear, Fisher Scientific, Cat. #NC9684584) with xylene and successive ethanol baths, followed by washing in PBST buffer (PBS plus 0.2% Tween 20). The slides were then processed to recover the antigenicity of 3B3hCC by using an antigen retrieval solution (BioGenex, Cat. #4K0869K) according to the manufacturer’s instructions. The slides were blocked with Power Block reagent (BioGenex, Cat. #HK085-5K) for 10 minutes at room temperature. They were then incubated with a polyclonal antibody against human IgG (Dako, Cat. #A0424) at 1:100 dilution overnight at 4°C in blocking buffer. After extensive washing with PBST, the slides were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Cat. #BA-1000) (1:100) for 30 min. After extensive washing with PBST again, the 3B3hCC antigen was visualized by
using an avidin-biotin-peroxidase conjugate (Vector Laboratories, Cat. #PK-4001). Color development was achieved by incubating the slide in AEC Peroxidase Substrate (Dako, Cat. #SK-4200). Images were obtained using a digital camera fit to a Zeiss Axioskop light microscope.

### 3.2.10 Monkey IFN-γ ELISpot assay

PBMC samples isolated from monkeys were assayed by ELISpot for cells which secrete IFN-γ. Sets of overlapping 3B3 peptides (each 18 amino acid peptides overlapping by 11 residues) were synthesized and purified by Genemed Synthesis, Inc. (San Francisco, CA). The preparation of peptide pools and monkey IFN-γ ELISpot assay were detailed in Chapter 2 Section 2.2.8. PBMC samples from each monkey were assayed in duplicate.

### 3.3 Results

#### 3.3.1 Construction of improved antibody genes to enhance expression

To achieve higher antibody expression levels in vivo, we reasoned that a stronger promoter, coupled with a more stable fusion partner, might result in higher serum concentrations possibly in a therapeutic range. With this goal in mind, we focused on the molecule scFv 3B3 that shares the Fab domains of IgG1b12. Using a similar strategy as employed with scFv X5, a codon-optimized cDNA encoding scFv 3B3 was cloned into plasmid pAAV/CMV.scFv X5 in place of scFv X5 (Figure 3.2). To increase scFv 3B3 half-life, we fused 3B3 with the rhesus heavy chain hinge, C2, and C3 domains (hCC) by overlap PCR yielding scFv 3B3hCC. The rhesus hCC was cloned from a cDNA preparation following RT-PCR using total RNA extracted from rhesus PBMCs. In a similar way, the scFv 3B3hCC was cloned into plasmid pAAV/CMV.scFv 3B3. DNA sequence analysis was performed to confirm the fidelity of the clones of pAAV/CMV.scFv 3B3 and pAAV/CMV.scFv 3B3hCC (see materials and methods).
In addition to the translational fusion, we wanted to determine whether alternative promoters could be used to further increase expression levels. Accordingly, 3B3 and 3B3hCC were cloned into a second AAV2 ITR based plasmid containing the CAG promoter. The CAG containing base plasmid was constructed by cloning of the CAG promoter from plasmid pCAG.Epo into plasmid pAAV/no promoter (C.2) (for details, see materials and methods Section 3.2.2).

In an attempt to enhance translational efficiency, the WPRE was attached after the stop codon of 3B3hCC or 3B3. The WPRE contains three cis-acting posttranscriptional regulatory elements, which may increase our scFv antibody expression. For purposes of comparison, we made only SS rAAV vectors for all the above constructs since the fragment of 3B3hCC (1.6 kb) plus the size of the CAG promoter (~ 2 kb) is over the packaging limit for a SC vector (Figure 3.2).

Lastly, to compare the effect of different serotypes, serotypes 1 and 8 were prepared by calcium phosphate transient transfection with AAV and Ad helper plasmids (pHelper, R88/C1 or R88/C8) (see materials and methods). pHelper plasmid contains genes from adenovirus required for AAV production (VA RNA, E2A, and E4). The R88 helper plasmid was recently developed by Dr. Bruce Schnepp in our laboratory and contains a portion of the viral ITR in a double-D conformation in addition to the rep and cap genes. The double-D conformation regulates rep and cap gene expression to near wild-type levels (Dr. Bruce Schnepp, personal communication). This improves production of rAAV without formation of detectable replication competent wt AAV. R88/C1 is a clone which encodes rep2/cap1 and R88/C8 encodes rep2/cap8. The viruses were purified using iodixanol gradient and column chromatography using PI POROS resin (for details, see materials and methods Section 3.2.3).
Vector genome titers were determined by a Q-PCR assay using primers and probes located in the CMV or CAG promoter region. As a result of QC testing, all vector stocks used in these experiments were determined to be sterile and possess low levels of endotoxin (63.3 EU/ml) (Figure 3.3: A). A typical purification result is shown in Figure 3.3 B, which revealed that vector stocks consist primarily of AAV capsid proteins with no detectable contaminants by SDS-PAGE analysis. Consistent with the virus being highly pure, no inflammation was observed after injection of the viral stocks into mice or monkeys.

3.3.2 Addition of hCC, WPRE, and use of serotype 8 dramatically enhance the expression of scFv antibody

In order to determine in vivo expression levels among the different vector configurations, we IM-injected Rag-1 mice with a dose of 10^{11} DRP/animal. The virus vectors contain CMV or CAG promoters, transgene 3B3 alone or 3B3hCC, serotype rAAV1 or rAAV8, with or without WPRE (Table 3.1). In general, the 3B3 or 3B3hCC expression levels in serum increased gradually with time and reached a steady state after week 4 (Table 3.1). Constructs containing 3B3 produced maximal levels between 0.02 and 1.0 \mu g/ml over the 16-week experiment. In contrast, constructs containing 3B3hCC produced maximal levels between 93 to 638 \mu g/ml depending on the particular configuration tested. Clearly expression levels between 3B3 and 3B3hCC were dramatically different with serum antibody concentration increased ~ 100 to 1,000 fold by the addition of the hCC fusion element (p < 0.001) (Figure 3.4 A). Analysis of the WPRE element demonstrated that this post-transcriptional sequence significantly increased 3B3hCC concentration by 4 to 8 fold over the constructs without the WPRE (p < 0.001) (Figure 3.4 B). Comparison of serotype revealed that AAV8 increased serum concentrations of 3B3hCC by approximately 2-fold over serotype 1 (p = 0.001) (Figure 3.4 C). No significant increase in
antibody expression was obtained using the CAG promoter in place of the CMV promoter (p > 0.05) (Figure 3.4 D).

In summary, the addition of heavy chain hCC fusion partner had the most profound affect consistent with a predicted increase in antibody half-life. Clearly, the fusion did not interfere with \textit{in vitro} binding activity. The WPRE provided a significant increase that also was consistent with the published AAV and retrovirus vector literature. Lastly, a modest increase in scFv levels was observed upon using the AAV8 capsid compared to the AAV1 capsid. In total, I was able to increase antibody expression at 400 – 6000 fold compared to the base scFv vector without the hCC fusion and WPRE element. The above results suggested that the optimal vector should be either rAAV8/CAG.3B3hCC.WPRE or rAAV8/CMV.3B3hCC.WPRE. However, since 40 - 80% of monkeys are infected with wild-type AAV8, and human sera demonstrated very little neutralizing activity to AAV8, we will continue to use rAAV1 for NHP studies. We remain open to the possibility of using rAAV8 for future human clinical trials (Gao, Alvira et al. 2002).

### 3.3.3 One site of scFv antibody expression

To demonstrate that muscle was the site of scFv 3B3hCC expression, paraffin-embedded Rag-1 mouse muscle tissue was stained for human IgG1. The left quadriceps was injected with rAAV1/CAG.3B3hCC at a dose of $10^{11}$ DRP in a 50 μl volume. Four weeks after injection, the muscle tissue was sectioned and stained. It demonstrated an appreciable, often punctuate immuno-staining of 3B3hCC (dark red color), which may represent secretory vesicles near the extracellular matrix surrounding muscle cells (Figure 3.5). The staining pattern tended to be distributed in the cytosol consistent with endoplasmic reticulum-Golgi localization of the secreted 3B3hCC. Similar staining was not detected in non-injected control right quadriceps tissue.
samples. This expression pattern was consistent with previous publications (Kay, Manno et al. 2000; Lewis, Chen et al. 2002). Therefore, the injected muscle was a site for transgene expression.

3.3.4 In vitro and in vivo molecular forms of scFv 3B3hCC

Like scFv X5, scFvs 3B3 and 3B3hCC were both driven by a single promoter in their respective rAAV constructs and were expressed as a single peptide (Figure 3.2). However, 3B3hCC may dimerize due to the inserted hinge region (Boadi, Schneider et al. 2005). To find out whether the 3B3hCC dimerized in vitro and in vivo, we performed western blot analysis on 3B3hCC expressed in both cell culture and mouse serum. 3B3hCC from mouse serum was isolated using immobilized Protein L beads. As Figure 3.6 shows, both cell culture and mouse serum purified 3B3hCC ran at about 100 kD without a reducing reagent (Figure 3.6: lane 3, 4), while the proteins migrated at 50 kD with inclusion of a reducing reagent (β-mercaptoethanol) (Figure 3.6: lane 6, 7). The dot-like band in lane 4 might be due to inefficient transfer from the SDS gel to the membrane. The 50 kD molecular weight (MW) band corresponds to the predicted size of 3B3hCC (i.e., 53 kD), while the 100 kD MW band is consistent with formation of a dimeric form (i.e., 106 kD). The top faint band in lane 6, which was from cell culture, could be from either a trimer of 3B3hCC or non-specific binding. These results imply that both in vivo and in vitro expressed 3B3hCC formed dimers efficiently since the 50 kD band was not observed under non-reducing conditions. This is significant because a bivalent molecule is likely not only to be more stable but also possesses greater avidity for the antigen compared to its monovalent counterpart (Bera, Williams-Gould et al. 2001; Roovers, van der Linden et al. 2001).
3.3.5 3B3 and 3B3hCC neutralization activity and heat stability

Although ELISA is a sensitive and rapid way to measure antibody binding to the gp120 monomer, an accurate measurement of biologic (neutralization) activity of each NAb is a better predictor of its therapeutic potential. Traditional neutralization assays were previously performed using infectious virus grown in primate PBMCs. Recently, a more sensitive and safer assay was developed by inserting a luciferase reporter gene in TZM-bl cells as described in Chapter 2. The assay measures neutralization by a reduction in luciferase gene expression using a luminescence-based assay. The luminescence intensity is directly proportional to the number of infectious virus particles present in the initial inoculum. Measurement of *E. coli* beta-galactosidase activity permits another way to accurately measure the infection. This assay is ideal to test the neutralization activities for many different compounds and alternative pseudotyped envelopes (Li, Gao et al. 2005; Montefiori 2005; Wang, Arthos et al. 2005; Gray, Meyers et al. 2006). Early on in our mouse experiments, our week 4 mouse sera, which contained high concentrations of 3B3hCC (~ 100 μg/ml), were assayed for neutralization activity using the TZM-bl based method. Surprisingly, no neutralization activities were detected against several pseudotyped viruses (SF162.LS and Du156.12), which the 3B3 scFv was known to neutralize (Miranda, Duval et al. 2007).

Based on the strong binding to gp120 in our ELISA, it was unclear why there was no neutralization activity in these mouse sera. I considered the possibility that the TZM-bl assay might not be measuring true neutralization activity, or the assay protocol was in some way interfering with neutralization measurement. Upon reviewing the assay protocol used in the Montefiori laboratory, I noticed that all serum samples were required to be heated at 56°C for one hour in order to inactivate the complement proteins present in the serum (Montefiori 2005).
Complement can interfere with the neutralization assay by lysing host cells or HIV-1 pseudovirions. The deposition of the C3b on the target cell or HIV-1 virion can activate the alternative complement pathway leading to cell lysis. Lysis of non-virally infected TZM-bl cells and HIV-1 could result in the generation of high assay background (B.D. Davis 1944; Frommhagen and Fudenberg 1962; Soltis, Hasz et al. 1979). While complement inactivation is a standard practice, it unfortunately may also irreversibly denature the scFv antibodies and cause them to lose their neutralization capabilities. This possibility became more understandable with the demonstration, by numerous laboratories, that scFv antibodies can have significantly different thermo-stability profiles compared to full-length antibodies (Dall’Acqua and Carter 1998; Willuda, Honegger et al. 1999; Orr, Carr et al. 2003; Brockmann, Cooper et al. 2005). Full length antibodies undergo phase transitions as seen by circular dichroism (CD) denaturing at temperatures over 60°C (Vermeer and Norde 2000), while scFv antibodies often show this phase transition between 50 - 55°C.

To test the hypothesis that the 3B3 scFv antibodies were heat sensitive at 56°C, I pooled week 12 mouse serum containing in vivo expressed 3B3hCC and performed a timed heat inactivation experiment at 56°C or 53°C for 15 to 60 minutes with one set of samples kept on ice. The samples were transferred to ice after heat and subsequently measured the binding ability for gp120 by ELISA. In a time dependent manner at 56°C for 15 minutes or more, serum samples containing 3B3hCC were observed to rapidly lose gp120-binding activity. This process was slowed by heating at 53°C (Figure 3.7: A). These data strongly suggested that 3B3hCC in serum becomes irreversibly denatured at 56°C resulting in failure to neutralize the pseudotyped viruses. To further address this issue, purified scFv 3B3 and 3B3hCC as well as IgG1b12 proteins were produced in cell culture of transiently transfected 293T cells in serum free media. The proteins
were purified from the media using immobilized protein L agarose beads according to the manufacturer’s protocol. Figure 3.8 shows a typical purification result for scFv 3B3. Surprisingly, purified scFv 3B3 demonstrated stable binding with gp120 in PBS after heating at 56°C for one hour (Figure 3.7 B). However, purified scFv 3B3 spiked into naïve mouse serum showed a similar heat denaturation profile as that of in vivo expressed 3B3hCC (Figure 3.7 A) and quickly lost activity. One possibility to explain this behavior was that 3B3 was being denatured reversibly in PBS, while in serum it was re-folded incorrectly in the presence of other serum proteins. In contrast, purified 3B3hCC gradually lost gp120-binding activity in both PBS and serum (Figure 3.7 C), but the process was slower in PBS than in animal serum. I next looked at the closely related full-length antibody IgG1b12 as an additional control. Purified IgG1b12 spiked into mouse serum (or PBS) did not lose any gp120 binding activity upon heating at 56°C for one hour (Figure 3.7 D). As expected, the full-length antibody is stable in serum which is consistent with the full-length antibody thermal stability data in the literature (Welfle, Misselwitz et al. 1999; Brockmann, Cooper et al. 2005). In conclusion, I have shown that the phase transition temperature of 3B3 and 3B3hCC occurs near 53°C and that at 56°C irreversible denaturation occurs in animal serum. These findings help explain the difference in the ELISA binding and neutralization data. While being significant for interpretation of our experiments, these temperatures are not physiologically relevant and should not impact the biological activity in vivo.

The neutralization assays were repeated without heat inactivation. Although the presence of serum complement has the potential to increase the background, which it did, robust neutralization activity in sera was observed after scFv gene transfer (Table 3.2). Although assay background was increased 2 to 5 fold, the neutralization activity correlated closely with scFv 3B3
or 3B3hCC expression levels. These results clearly demonstrate that rAAV mediated gene transfer of the 3B3hCC moiety could impart high-titer neutralization activity to the host animal and muscle expressed antibodies retain predicted neutralization activity.

### 3.3.6 NHP challenge study with rAAV1/CAG.3B3hCC

Based on the mouse expression data, four macaque monkeys were injected with $3.5 \times 10^{13}$ DRP of SS rAAV1/CAG.3B3hCC intramuscularly ($4 - 6 \times 10^{12}$ DRP/kg). The animals were bled biweekly and the concentrations of 3B3hCC were measured by gp120 ELISA. As Figure 3.9 shows, high levels of 3B3hCC were achieved in week 2 in all four animals (M1 - RQ6611, M2 - RQ6613, M3 - RQ6638, M4 - RQ6639). Monkeys M3 and M4 possessed ~ 33 μg/ml, while monkey M2 had 20.6 μg/ml levels and monkey M1 reached 14.5 μg/ml. However, only monkey M3 had persistent expression over a four month time period, while 3B3hCC concentrations in M1, M2, and M4 dropped sharply after week 2. Significantly, neutralizing activity against a CCR5 utilizing HIV-1 isolate (SF162.LS) in these monkey sera were detected at weeks 2 and 4, which is consistent with the gp120 binding data (Table 3.3). Similar to the mouse sera, neutralizing activities in sera were detected without heat treatment. As expected, neutralizing activities closely correlated with 3B3hCC concentrations (Figure 3.9). In addition, neutralizing activities against Du156.12 were also detected in these sera (Table 3.3). These data clearly document that rAAV1 administration could endow rhesus macaques with *in vitro* neutralization activity after a single intra-muscular injection. Also, the expressed antibody showed neutralization activity consistent with the 3B3 molecule.

Since monkey M3 reached serum levels close to the threshold therapeutic range (based on passive immunization studies) at week 2 post-injection, the four vaccinated monkeys, as well as four
naïve control monkeys, were challenged with SF162P3 SHIV virus at a dose of 50 MID\(_{50}\) by the intra-rectal route at week 4. After challenge, monkey M3 maintained significant 3B3hCC levels, while the other animals demonstrated various rates of decline. M1 levels were undetectable, while animals M2 and M4 levels stabilized in the range of 1 - 5 \(\mu\)g/ml. Unfortunately, all four vaccinated monkeys were infected by the challenge inoculum based on the viral load results (Table 3.4). The incremental increase of 3B3hCC levels in monkey M4 after week 14 might be due to host antibodies elicited as a result of viral infection. Statistically, there was no significant difference between the control group and vaccinated group in viral load at all the weeks measured (student t-test, \(p > 0.05\)). Therefore, no protection against SHIV infection was observed in any of the vaccinated animals despite low to modest expression of the 3B3hCC antibody.

3.3.7 rAAV vector persistence at site of injection

Why did the four monkeys demonstrate such dramatic differences in antibody expression? If host immune responses in monkeys M1, M2 and M4 caused clearance of vector transduced myocytes and, therefore, resulted in loss of gene expression, we reasoned that the muscle tissue in these 3 animals should have lower amounts of vector DNA than in monkey M3. In order to test this, rAAV1/CAG.3B3hCC genomes were measured by Q-PCR in muscle DNA extracted from harvested tissue obtained at necropsy 46 weeks post vaccination (42 weeks post challenge). rAAV1/CAG.3B3hCC vector DNA copy numbers were measured using real-time Q-PCR whereby the quadriceps muscle was divided into 9 equal sections (Figure 3.10 diagram) and total DNA isolated and subjected to TaqMan Q-PCR using a PCR primer-probe pair specific for the CAG promoter. As shown in Figure 3.10, all the injected animal’s muscle tissue possessed significant levels of vector DNA scattered in different sections. Naïve control monkey tissues were negative for vector DNA (M0). Maximal vector DNA was found in sections 8 and 9 from
monkeys M1 and M2, while vector DNA was concentrated in sections 5 and 6 in monkeys M3 and M4. The total vector DNA copy numbers ranged between 5 and 35 copies per nucleus, with M3 having the highest total vector copy number followed by monkeys M1, M2, and M4 with the least. Thus, vector genomes were found in all the vaccinated monkeys at relatively high levels in localized areas with vector transduction levels loosely correlating with expression levels in the blood in monkeys M2, M3, and M4 but not in monkey M1.

### 3.3.8 Humoral and cellular responses against the transgene and vector

While we did not observe a direct correlation between the genome copy number and 3B3hCC expression levels for all animals, it seemed logical to characterize the host immune responses against the vector and transgene to see if either antigen was triggering a host immune response that impacted scFv expression. During construction of scFv 3B3hCC, several new epitopes may have been created from the cloning process including: 1) the gly-ser linker region between V\textsubscript{H} and V\textsubscript{L}; 2) the region between the hinge and V\textsubscript{L}; and 3) possibly, secondary and tertiary changes since the CH1 and C\textsubscript{L} domains were omitted. Therefore, portions of 3B3hCC besides the human version of 3B3 could potentially be immunogenic, which could affect its persistence and \textit{in vivo} expression levels. In addition, the AAV1 capsid is a particulate antigen that has been shown to readily stimulate humoral and cell mediated immune responses in multiple animal species including humans (Nathwani, Davidoff et al. 2002; Manno, Pierce et al. 2006).

#### 3.3.8.1 Humoral immune responses

To find out whether antibodies against the transgene were produced, sera from the four vaccinated monkeys collected from weeks 2 through 14 were subjected to western strip blot
analysis. Purified 3B3 was used as the target antigen and run on SDS-PAGE gels and transferred to a Hybond-P membrane. Diluted primate serum samples (1:100) were used as the primary antibody and incubated with the 3B3 antigen that was separated into distinct lanes to allow for multiple sample incubations. Sera prior to vaccination served as negative controls. Following washing, the blot was then incubated with an anti-monkey IgG-HRP conjugated secondary antibody. As shown in Figure 3.11, monkey M1 produced antibodies against 3B3 starting as early as 6 weeks post-vaccination, which increased gradually to a peak level at week 12. Monkey M2 produced high-levels of anti-3B3 antibodies only at week 14, while the other two animals, M3 and M4, did not show any anti-3B3 antibodies from weeks 2 through 14. It is most likely that the anti-3B3 antibodies detected are against variable domains of 3B3, therefore they should be called anti-idiotypic antibodies. These data demonstrated variable and modest anti-transgene antibody responses that overall did not appear to correlate with the observed early drop in 3B3hCC levels observed in monkeys M2 and M4. Only animal M1 had a temporal profile that could possibly relate to the loss of the transgene expression.

To characterize the humoral immune response against AAV1, a solid phase ELISA was developed that consisted of coating wells with purified AAV1 and detecting the presence of anti-AAV1 binding antibodies in animal serum. As Figure 3.12 shows, three animals were clearly positive for antibodies against AAV1 before rAAV1 injection, indicating the presence of pre-existing exposure to AAV in these animals (M1, M2, and M4). Monkey M3 possessed a borderline response at the lowest serum dilution assayed (1:100). As expected, all animals had an increased humoral response following rAAV1 vector administration by 4 weeks post-vaccination that was maintained (Figure 3.12). Interestingly, monkey M3, which may have not been previously exposed, correlated with the observed highest expression levels of 3B3hCC. We note
however that none of the animals had a detectable anti-capsid cellular response which would be expected if previous exposure was driving the loss of gene expression. Regardless, our data suggests that pre-screening animals for lack of binding AAV antibodies may be wise since this is consistent with identification of animals that have not been exposed to AAV by natural infection.

### 3.3.8.2 Cellular immune responses against the transgene and vector

To determine whether a cellular immune response against the transgene or vector was present in any of the four experimental monkeys, frozen PBMCs at weeks 0, 2, 4, and 8 were subjected to the ELISpot assay after *in vitro* stimulation with overlapping peptide pools based on AAV1 VP1 and 3B3 (scFv portion only) protein sequences. To control for non-specific stimulation of PBMCs, peptide pools based on the eGFP and PBMCs before vaccination were used as negative controls. For the positive control, ConA was used which is a lymphocyte mitogen that stimulates lymphocytes to secret IFN-γ. All monkey PBMCs from weeks 2 - 8 were found to be negative in the IFN-γ ELISpot assay when stimulated with either vector capsid or transgene pools. ConA wells had more than 1,000 SFCs/million PBMCs, which was as expected. Wells stimulated with the eGFP peptide pool (Pe) were also negative. Figure 3.13 shows representative IFN-γ ELISpot data for monkey M1. We acknowledge that overlapping peptide pools from the 3B3 sequence did not cover every possible epitope occurring in the protein 3B3hCC and, therefore, we may have missed responses to neo epitopes or those in the rhesus macaque hCC region. Additionally, we used frozen PBMCs for these experiments and it is appreciated that this reduces assay sensitivity compared to the use of freshly isolated PBMCs. In conclusion, no evidence for capsid or 3B3-specific T cell responses was found in the four vaccinated monkeys.
3.4 Discussion

3.4.1 Improvement through use of a genetic fusion partner, WPRE, and serotype 8

The goal of these studies was to increase the serum concentration of an anti-HIV-1 neutralizing antibody into a possible therapeutic range. Our rodent data showed that use of a genetic fusion protein dramatically improved mouse serum concentrations by 100 to 1000 fold over the base scFv. The inclusion of a WPRE element further improved the mouse serum concentrations 4 to 8 fold. Furthermore, use of an alternative serotype (AAV8-like AAVrh.74) mediated a 2-fold increase in serum levels. Consistent with the mouse data, 3B3hCC peak concentrations in these four monkeys ranged 10 to 100 fold higher than that detected for scFv X5 in 2 NHPs receiving the same dose of rAAV1. Moreover, the vector rAAV1/CAG.3B3hCC endowed four NHPs with detectable neutralization activity against a primary-like HIV-1 envelope virus.

Our ability to increase serum antibody concentration was made possible by combining three distinct improvements: use of a genetic fusion partner, WPRE element, and use of an alternative serotype. We exploited a cloned antibody fragment with predetermined binding specificity without steric inhibition and fused it to the constant region of macaque IgG1. The IgG1 constant region allowed the scFv 3B3hCC to dimerize in vitro and in vivo, which may have multiple benefits in terms of avidity and stability (Afanasieva, Wittmer et al. 2003; Hudson and Souriau 2003). Secondly, the WPRE increased the antibody concentration to a similar extent as previously reported in retro/lentiviruses, adenoviruses and AAV (Zufferey, Donello et al. 1999, Appleby, Kingston et al. 2003). The WPRE most likely functions within the cell nucleus to increase the levels of nuclear transcripts by increasing the efficiency of RNA processing and preventing RNA degradation (Donello, Loeb et al. 1998).
The serotype 8 AAV isolate (rh.74) was obtained here at NCH by Dr. Ryan Jensen from rhesus monkey lymph node. Our research group has shown that it readily crosses the vascular endothelium, which may be beneficial for systemic gene transfer (Rodino et al. 2007). AAV8 has also been shown to mediate higher transgene expression compared to rAAV1 and rAAV2 in liver, pancreas, and brain following systemic delivery (Gao, Alvira et al. 2002; Nakai, Fuess et al. 2005; Wang, Zhu et al. 2005; Cheng, Wolfe et al. 2007). Serotype 8 uses LamR, a receptor for laminin, prions and some other viruses, which is widely expressed in human tissues such as liver, heart, and skeletal muscle. Notably, LamR is also over-expressed in tumor cells and, therefore, this serotype might also work well for tumor-directed gene therapy (Akache, Grimm et al. 2006).

3.4.2 Heat stability of scFv antibody

Normal IgG undergoes two main independent transitions upon thermal denaturation – each representing the unfolding of the Fab and Fc fragments, respectively, when analyzed by differential scanning calorimetry (DSC) and/or circular dichroism (CD) (Vermeer and Norde 2000). The Tms from the two transitions correspond to the thermal stabilities of the Fab and Fc domains, with the Fab domain Tm (first peak in DSC) almost always greater than 60°C, while the Tm of the Fc domain is usually greater than 70°C under physiological conditions (Vermeer and Norde 2000). When heated over 60°C, IgG may also undergo irreversible aggregation if the protein concentration is high (2 - 20 mg/ml) or in the presence of other serum proteins (Rosenqvist, Jossang et al. 1987; Vermeer and Norde 2000). Therefore, heating serum at 56°C to inactivate complement does not inactivate most IgGs. Based on our data and the published literature, it appears that scFvs have lower Tms than that observed for native antibodies. Apparently, irreversible denaturation of the scFv domain in 3B3 and 3B3hCC abolished their gp120 binding capacities and therefore destroyed their neutralization activities. Consistent with
antibody aggregation, we observed that the scFv antibodies in serum were less active than the purified proteins in PBS (at least for 3B3). Detailed DSC and/or CD experiments on these purified scFv antibodies are needed to further support this hypothesis but similar analyses with other scFv molecules have indeed demonstrated reduced thermal transition temperatures with the portion of scFvs.

### 3.4.3 3B3hCC levels compared to published therapeutic levels

Passive immunization studies suggested that plasma NAb levels between 50 - 200 μg/ml at the time of virus challenge are sufficient to provide significant protection in a significant proportion of vaccinees, and result in reduced viremia and increased CD4+ levels in those that do become infected (Mascola, Louder et al. 1997; Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999; Mascola, Stiegler et al. 2000; Mascola, Lewis et al. 2003). For monkey M3, a neutralizing antibody concentration of 43.0 μg/ml, at the time of challenge, is close to the minimal therapeutic level predicted from passive immunizations. However, we observed no protection in this animal. We note that 3B3 has a higher affinity for gp120 than b12, and, therefore, the 3B3hCC serum concentration calculated by using an IgG1b12 standard may overestimate its actual levels. (Roovers, van der Linden et al. 2001; McHugh, Hu et al. 2002). Clearly, with this challenge virus, dose, and route, no evidence of protection in any monkey was observed.

### 3.4.4 Viral vector biodistribution

Although the same dose of viral vector was injected in the same manner to each of the four monkeys, the expression patterns and levels were different. Only M3 had persistent gene expression over 4 months while the other three monkeys showed sharp drop offs after week 2. Monkey M1 dropped to undetectable levels after week 6 and monkeys M2 and M4 maintained
levels of about 1 to 5 μg/ml over six months. Although there are some local differences, all animals had certain amounts vectors in the injected muscle tissues detected by Q-PCR after the animals were sacrificed. These data were not consistent with their expression levels, especially for monkey M1. Monkeys M2 and M4 had lower genome copy numbers, which correlated to their lower expression levels after week 6. These results suggested that some of the transduced cells might have been cleared and/or inhibited by an immune response as indicated in rAAV2 mediated liver gene transduction in humans (Mingozzi, Maus et al. 2007). Monkey M1 had relatively high genome copy number while its expression was almost completely lost after week 4, which was most likely masked by anti-idiotypic response against 3B3 (next section). Another alternative possibility is that the transgene expression from the transduced cells was in some manner blocked possibly by promoter shutdown.

3.4.5. Humoral and cellular immune responses to the transgene and vector

Generally, human immunoglobulins share a high degree of homology with their primate counterparts, including variable regions (Andris, Miller et al. 1997; Calvas, Apoil et al. 1999). Consistent with the high degree of sequence identity, western-blot analysis revealed that only monkey M1 produced persistent anti-idiotypic antibodies, while monkey M2 produced significant high level of anti-antibodies only at late week. These data suggested that the human 3B3 portion induced a variable immunogenic response in these rhesus macaques. Of course, using purified 3B3 instead of 3B3hCC to detect the anti-antibody against 3B3hCC in monkey serum might have missed antibodies against novel epitopes in the constant and/or junction regions. However, for monkey M1, this result help to explain why the monkey had high vector copy number while the gene expression was completely lost after week 4. Since the anti-antibodies were against 3B3 portion, the expressed 3B3hCC were most likely masked by these anti-antibodies. The reason
why we did not observe any anti-3B3 at week 4 was that the western blot only detected IgG class since the secondary antibody was anti-human IgG (refer to the material and method section). It is generally believed that IgM is the first isotype of antibody synthesized and secreted during the primary humoral response followed by IgG and IgA. More experiments are needed to clarify this situation. A similar mechanism will be discussed in Chapter 4.

With regards to the capsid, although rAAV does not encode any viral protein, the capsid proteins themselves do stimulate host immune responses. We observed robust anti-AAV binding antibodies in all animals, which was expected. The major consequence of this response is the inability to re-dose animals with the same serotype (and possibly other related serotypes) due to the presence of neutralizing antibodies. Our anti-AAV ELISA analysis was consistent with the view that pre-existing humoral immunity against AAV might be an important factor to consider when performing gene transfer studies in the primate host that undergoes natural infection. We noted that the levels of anti-AAV1 antibodies before injection correlated with long term 3B3hCC expression levels. This result is consistent with Jim Wilson lab’s observation, in which pre-existing immunity (NAbs) against the rAAV vector substantially diminished rAAV-mediated transgene expression in liver (Gao, Lu et al. 2006). AAV sero-positivity, if problematic in the general population, may be overcome through the use of highly divergent serotypes from those which circulate in the population (AAV2, 3, and 2/3 hybrid). In addition, human seroconversion occurs with age and, therefore, younger children show lower levels of seropositivity with peak levels occurring in early adolescence. Accordingly, it will be ideal to vaccinate younger children with rAAV-mediated vaccines.
As in the scFv X5 monkey study, we did not find evidence for cellular responses targeting the AAV1 capsid or 3B3 portion of the molecule. Overlapping peptide pools from 3B3 did not contain every epitope present in 3B3hCC and may have missed novel epitopes in the hC2C3 region. It is also possible that our ELISpot sensitivity was too low to detect antigen specific responses since we used frozen PBMCs instead of fresh or expanded PBMCs as employed in several recent studies (Casimiro, Wang et al. 2005; Zhang, Huang et al. 2006; Minozzi, Maus et al. 2007). Studies in the first clinical trial of hepatic AAV2 factor IX gene transfer demonstrated that some human subjects carrying AAV2-specific memory CD8+ cells at frequencies that are commonly too low for detection by conventional direct in vitro ELISpot methods (Mingozzi, Maus et al. 2007). In vitro stimulation with a single peptide requires a frequency of memory T cells at or above 1 in 10⁶ PBMCs. Levels of memory T cells lower than that will escape detection even upon cell expansion. A more sensitive detection method may be needed. In conclusion, cellular immune response against 3B3hCC and the capsid, which might clear 3B3hCC transduced cells, although not detected, cannot be excluded.

3.4.6 Can we improve upon the challenge model?

Although monkeys M1, M2, and M4 had lower concentrations of 3B3hCC at the time of challenge (week 4), monkey M3 had approximately 43 μg/ml of 3B3hCC. Then why did we not obtain any evidence of protection or blunting of replication in this animal? In addition to possibly overestimating levels because we used IgG1b12 as the standard, other possibilities are discussed in the following. First, the challenge route, although relevant, might have been a problem. There are five ways that are commonly used to challenge a monkey with SIV/SHIV. The challenge routes can be divided into two categories: one is mucosal surface that includes intra-rectal, oral,
urethral, and vaginal challenges and the other is by intravenous challenge. Intravenous challenge is the most commonly used method and usually a dose of 10 to 100 MID₅₀ is needed for a successful challenge (Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999). Mucosal membrane challenges require higher doses of challenge virus but our dose of 50 MID₅₀ was clearly sufficient to infect all eight animals including 4 naïve control monkeys from Chapter 2. The intravenous challenge-model mimics infection by blood transfusion but HIV-1 is most often transmitted through mucous membranes of the vagina or rectum (Boadi, Schneider et al. 2005; Martinon, Brochard et al. 2008). In the later case, both male and female monkeys can be studied. Significantly, using similar neutralizing antibodies and challenge virus, passive immunization studies demonstrated that greater protection was achieved against a mucosal challenge compared to an intravenous challenge (Mascola, Lewis et al. 1999; Mascola, Stiegler et al. 2000; Parren, Marx et al. 2001). This was our rationale for the intra-rectal route. IgG (and scFvs) are serum proteins that are present in the blood that can transudate across epithelium and protect against mucosal respiratory infections. Passive immunization studies where 100 mg to 1 g of purified IgG1b12 was infused into rhesus macaques possessed only 100 to 900 μg/ml in serum, and concentrations on local mucosal membrane surfaces such as vaginal fluids were 20 to 30 times lower than that in serum 24 hours post-administration (Mascola, Stiegler et al. 2000). Since the local antibody concentration is a critical factor, it may be more logical to test our system using an IV challenge where our NAb is readily accessible.

Mouse model and in vitro experiments using a modified organ culture system demonstrated that dendritic cells (DC), Langerhans cells (LC), and intraepithelial CD4+ T cells are the initial targets for HIV-1 infection (Masurier, Salomon et al. 1998; Salomon, Cohen et al. 1998). Infected DCs and LCs carry the virus to draining lymph nodes (LN). From LNs, the virus spreads into the
blood reaching the systemic lymphatic, including the GALT. NAbs work by blocking initial HIV-1 infection to achieve sterilizing immunity or by partially blocking the initial infection to achieve a lower viral set point by containing the virus in the regional lymphatics near the portal of entry. Set point is an average value of viral load of an infected host during the asymptomatic stage (Fraser, Hollingsworth et al. 2007). Once a productive HIV-1/SIV infection is established, NAbs cannot eradicate the infection, but may improve the health of the infected animal, to some extent, by controlling viremia (Haigwood, Watson et al. 1996; Haigwood, Montefiori et al. 2004). To target the mucosa, one possibility is to fuse a scFv with the hCC domain from the heavy chain of IgA, which is secreted mucosal antibody. The key would be to obtain localized mucosal production of the IgA construct. However, to date, AAV serotypes appear to poorly transduce the mucosal epithelium and long-term expression may be problematic due to cellular turnover.

Secondly, dose and injection sites can also be optimized. In Chapter 2, we tried to define an upper dose limit for muscle injection in our Rag-1 mouse model. We did not find one for rAAV1/CMV.scFv X5. However, too many vector genomes in one single cell may saturate circularization and secretion pathways, possibly leading to apoptosis. Therefore, an appropriate dose maximizes delivery by not saturating expression pathways or cellular receptors. Unfortunately, this requires empirical dose-escalation studies that remain prohibitively expensive in the macaque model. In our 3B3hCC monkey study performed here, we used the same dose of the virus as that in scFv X5 studies. The serum 3B3hCC levels dropped significantly after week 2 in three of the four monkeys and the possible influence of a high bolus dose per site cannot be excluded as a factor that mitigates long-term expression. Arruda et al. recommend dosing in the range of 2 - 5x10^{12} DRP/kg per site by intra-muscular injection (Arruda, Schuettrumpf et al. 2004). On the other hand, too many injection sites may not be practical for a preventive vaccine.
3.4.7 Immune responses

As discussed in Section 3.4.4, immune responses against the transgene could potentially be a problem since scFv 3B3 was derived from human DNA sequences and several new epitopes may have been created during the cloning process. Although we did not find any cellular response against 3B3 and only detected a humoral immune response against 3B3 in two of the four monkeys, it does not mean that a cellular immune response against 3B3hCC did not exist. Technical limitations may be important factors. For the vector capsid, since three out of four animals had pre-existing antibodies against AAV1, this indicates natural infection with wild-type AAV occurred. Therefore, the possibility of a capsid-specific immune cells cannot be excluded. To better prove the concept of the rAAV mediated gene transfer in the NHP model, it seems reasonable to think of working with all simian antibody genes in AAV naïve animals to remove these caveats from the discussion. This approach is more fully explored in the next chapter.

In summary, three combined approaches: genetic fusion, including an WPRE, and using AAV serotype 8, could potentially increase in vivo antibody levels by 400 – 12,000 fold over the initial base scFv construct corresponding to steady-state antibody concentrations in the range of 200 – 1200 μg/ml in mice. Fusion with IgG1 hCC alone increased transgene levels by 100 - 1,000 fold in mice and NHPs. We demonstrated that high-titer neutralizing activity against the R5 utilizing HIV-1 isolate SF162 was achieved following antibody gene transfer in 4/4 macaques providing initial proof-of-concept data in this large animal model. Evidence for a persistent anti-antibody response was found in only one primate (M1) that appeared to block gp120 binding activity. Humoral immune responses against vector and transgene were documented and pre-existing AAV immunity may have played a role in limiting the duration of gene expression, although no direct data supporting this idea was obtained. Several experimental modifications are now
planned based on our experience with this challenge study including: dose, transgene, route, vector design, pre-existing AAV sero-status and challenge virus.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Animal ID</th>
<th>Weeks</th>
</tr>
</thead>
<tbody>
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<td>Control (PBS)</td>
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</tr>
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<td></td>
<td>B1g</td>
<td>0.00 0.00 0.00 0.00 0.00 0.00 0.00</td>
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<td>RT021g</td>
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<td>H2g</td>
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<td>I2b</td>
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<td>J1g</td>
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<td>M2b</td>
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<td></td>
<td>N2g</td>
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<td>RT61g</td>
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<td>RT62g</td>
<td>0.00 92.0 179.9 292.7 169.2 265.0 251.7</td>
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</table>

Note: b: male mouse, g: female mouse, all the mice were injected in the left leg with 1.0x10^{11} DRP virus in 50 μl volume, while the control mice were injected with PBS buffer. NA: data not available.
Table 3.2: Neutralization activity of pooled mouse sera with and without pre-heating

<table>
<thead>
<tr>
<th>Animal group/ Virus construct</th>
<th>Bleed date</th>
<th>Bleed Week</th>
<th>SF162.LS (No heat)</th>
<th>SF162.LS (Heat at 56°C)</th>
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<td>GH rAAV1/CAG.3B3</td>
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<td>121</td>
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<tr>
<td></td>
<td>03-04-05</td>
<td>4</td>
<td>137</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>04-04-05</td>
<td>8</td>
<td>105</td>
<td>&lt;20</td>
</tr>
<tr>
<td>IJ rAAV1/CAG.3B3hCC</td>
<td>02-01-05</td>
<td>0</td>
<td>121</td>
<td>&lt;20</td>
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<td></td>
<td>03-04-05</td>
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<td>1,362</td>
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<td></td>
<td>04-04-05</td>
<td>8</td>
<td>2,400</td>
<td>&lt;20</td>
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<tr>
<td>EF rAAV1/CMV.3B3hCC</td>
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<td>88</td>
<td>&lt;20</td>
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<td></td>
<td>03-04-05</td>
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<td>1,951</td>
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<td>04-04-05</td>
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<td>4,005</td>
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Note: values are the serum dilution at which relative luminescence units (RLU) were reduced 50% compared to virus control wells (no test sample). Each data was obtained using pooled serum samples from four individual mice since the assay required large amount of samples. Group GH includes animals G1b, G2b, H1g, and H2g; IJ includes I1b, I2b, J1g, and J2g; EF includes E1b, E2b, F1g, and F2g (Table 3.1). At the condition of no heat, the complement was present without adding any inhibitors.
Table 3.3: Neutralization activity of vaccinated rhesus macaques

<table>
<thead>
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<th>Animal</th>
<th>Date</th>
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Note: values are the serum dilution at which relative luminescence units (RLU) were reduced 50% compared to that from virus control wells. For the control protein IgG1b12, the data shows the protein concentration needed for a 50% reduction of the RLU compared to that from virus control wells (IC50).
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Figure 3.1: ELISA measurement of transgene expression from clones of X5 fusion with A1AT. C12 cells were transiently transfected with pAAV/CMV plasmid containing either X5 only, A1AT only, X5-A1AT, or A1AT-X5 in a six-well format. Plasmids containing X5 and A1AT only serve as controls. The cell media were harvested 48 hours post transfection. The transgene expression was measured by either a gp120 ELISA to detect X5 portion of the expressed protein (on the left of the figure) or A1AT ELISA to detect A1AT portion of the expressed protein (on the right of the figure) at dilution of 1:20. Gp120 ELISA for X5 measurement was described in Chapter 2. A1AT ELISA for A1AT measurement was described previously (Song, Morgan et al. 1998). An OD450nm value of 4.0 indicates saturation of the color reaction in ELISA.
Figure 3.2: Schematic diagram of pAAV/CMV.3B3 and pAAV/CMV.3B3hCC. The structures of SS vectors rAAV/CMV.3B3 and rAAV/CMV.3B3hCC are shown. Each expression cassette was cloned between two ITRs. Synthetic leader sequence was incorporated before the V\textsubscript{Light} chain indicated as a right-angled arrow; GS: (4GS)\textsubscript{3} peptide linker for V\textsubscript{Heavy} and V\textsubscript{Light}; H: Hinge region; C2 and C3: Constant region of IgG1. ITRs are shown as arrowheads.
A:

Viral Vector Core Laboratory

Research

Order ID: FO83

Vector: rAAV8.CAG.3B3HCC

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B:

F083 Control
Figure 3.3: The certificate of analysis of vector preparation for the vector rAAV8/CAG.3B3hCC (F083). A: The certification and the quality control results. B: SDS-PAGE analysis of the purified vector. rAAV8/CMV.eGFP was used as a control. 20 μl of each virus was loaded on a 10% SDS gel. Capsid proteins (VP1, VP2, VP3) are labeled on the figure. The gel was fixed in 7.5% acetic acid solution and stained by SYPRO Orange dye.
Figure 3.4: Serum concentrations of scFv 3B3 and 3B3hCC in mice. Six to eight week old Rag-1 mice were injected with different rAAV virus at a dose of $10^{11}$ DRP/animal. Mice injected with PBS served as controls. Each group included four animals with two males and two females ($n = 4$). The mice were bled biweekly. Serum concentrations of scFv 3B3 and 3B3hCC were quantitated by an HIV-1 gp120 binding ELISA using a known IgG1b12 standard. “1” and “8” before CMV or CAG represent the AAV serotype. A: serum concentrations comparison among constructs with and without hCC; B: serum concentration comparison between 3B3hCC constructs with and without WPRE; C: serum concentration comparison between 3B3hCC constructs packaged by serotype 1 or 8; D: serum concentration comparison between 3B3hCC constructs driven by CMV or CAG promoter.
Figure 3.5: Muscle as a site for antibody production. Human IgG1 immunohistochemistry was performed on 4 μm paraffin sections of the injected left quadriceps muscle (LQ - bottom panel) and un-injected right quadriceps (RQ - top panel) from a mouse receiving rAAV1/CMV.3B3hCC 12 weeks post-injection. Detection was accomplished using HRP-conjugated anti-human IgG1, followed by color development with the AEC substrate (red). Cells were counter stained with hematoxylin (blue).
Figure 3.6: Western blot analysis of scFv 3B3hCC expressed in vitro and in vivo. Samples of scFv 3B3hCC, either in cell culture media or purified from mouse sera, were run on a 7.5% SDS-PAGE. The proteins were then transferred to a Hybond-P membrane and hybridized with protein L HRP conjugate (1:5,000). Lane M: 10 μl Magic Mark XP; lanes 2, 5: control sample purified from naïve control mouse serum at week 6; lanes 3, 6: 10 μl 3B3hCC from cell culture media (7 μg/ml); lanes 4, 7: 0.5 μl purified 3B3hCC from 100 μl mouse serum at week 6 injected with rAAV1/CAG.3B3hCC; lanes 5, 6, 7: samples contained 5% reducing reagent β-mercaptoethanol.
Figure 3.7: Heat inactivation of scFv 3B3 and 3B3hCC in animal serum and PBS buffer. OD450nm values from the ELISA (see materials and methods) are plotted versus the time in minutes at the indicated temperatures. A: serum containing 3B3hCC from an injected mouse at week 12 was incubated at 53°C or 56°C. B-D: purified protein either 3B3 (B), 3B3hCC (C), or IgG1b12 (D) were incubated in PBS or Rag-1 mouse serum (1:10 dilution) at 56°C. After heating at the indicated times, the samples were placed on ice. The OD450nm values were measured by the gp120 ELISA on these samples after dilution in PBST.
Figure 3.8: SDS-PAGE analysis of scFv 3B3 protein purification. 100 ml of cell supernatant was mixed with immobilized protein L beads (1 ml) at 4°C overnight. The beads were then packed in a column for washing and elution. The column was washed with PBS and the 3B3 protein eluted in low pH IgG elution buffer. Lane 1: cell supernatant; Lane 2: cell supernatant after passing the column; Lanes 3, 4, 5, 6, 7: sequential PBS washes (5, 10, 15, 20, 25 ml respectively); Lanes 8, 9, 10: elution 1, 2, 3 (2 ml each).
Figure 3.9: scFv 3B3hCC serum concentrations in vaccinated rhesus macaques. Four random selected monkeys were injected with $3.5 \times 10^{13}$ DRP rAAV1/CAG.3B3hCC virus intramuscularly. The monkeys were bled biweekly. scFv 3B3hCC antibody serum concentrations were quantitated by a gp120 binding ELISA method using a known antibody standard IgG1b12. The four monkeys were challenge at week 4 post vaccination as indicated in the figure (DOC, day of challenge).
Figure 3.10: Viral vector biodistribution. For each monkey, the entire muscle where the injection was performed was harvested and divided into nine sections: 1, 2, 3, 4, 5, 6, 7, 8, and 9. Total DNA was extracted from each tissue block and the relative copy number was calculated according to Q-PCR data. The “section 10” is the total number for all the sections for that monkey. M0 is a naïve control monkey.
Monkey: RQ6611(1), RQ6613(2), RQ6638(3), RQ6639(4)

RQ6606(C1), RQ6609(C2)

Figure 3.11: Western strip blot detection of anti-3B3 humoral response. Purified 3B3 antigen was run on a SDS gel and a western blot was performed using monkey serum from the indicated individuals and times. The top and bottom integer numbers were serum samples from the corresponding monkeys. 1 represents monkey RQ6611, 2 monkey RQ6613, 3 monkey RQ6638, and 4 monkey RQ6639. C1 and C2 were naïve control monkey sera from monkeys RQ6606 and RQ6609, respectively. M: Magic Marker XP.
Figure 3.12: Humoral immune response against AAV1 detected by ELISA. OD450nm values from the ELISA are plotted versus the different week serum samples of monkeys M1, M2, M3, and M4. W0 was pre-bleed before the vector injection. All the serum samples were diluted 1:100 in PBST before binding to a coated and blocked ELISA plate.
Figure 3.13: Number of spot forming cells per million PBMCs from monkey M1 at different weeks. W0 was the pre-bleed before the vector injection. PBMCs were isolated by Ficoll-Pacque density gradient centrifugation. ConA, a lymphocyte mitogen, stimulates lymphocyte to grow. All the wells stimulated by ConA had more than 1,000 SFCs/10^6 seeded PBMCs, which were used as positive controls.
CHAPTER 4

IN VIVO GENE TRANSFER PROVIDES PROTECTIVE IMMUNITY AGAINST VIRULENT SIV CHALLENGE IN A RHESUS MACAQUE INFECTION MODEL

4.1 Introduction

Our previous experiments using SC rAAV1/CMV.scFv X5 and SS rAAV1/CAG.3B3hcc in rhesus macaques resulted in neutralizing antibody levels that were not high enough to obtain benefits against challenge viruses. Possible reasons include: a short half-life of the molecule; pre-existing wtAAV immunity; possible immune responses against the human proteins; vector dosage/site used; route and virus isolate used for challenge. To address these challenges and provide proof-of-concept for antibody gene transfer using the rAAV delivery platform, we expanded our approach to use: 1) a fusion strategy using an antibody Fc region to increase the scFv’s half-life as demonstrated in Chapter 3; 2) monkeys which have no or low titers of pre-existing anti-AAV antibodies; 3) entry inhibitory molecules derived from rhesus sequences to lower the possibility of immune responses against the transgene; 4) lower vector dosages per site and more injection sites; and 5) an IV challenge with a pathogenic strain of SIV.
4.1.1 Overview of NAbs against different SIV strains

Since the discovery of SIV from *Macaca mulatta* and its induction of AIDS-like illness in macaques, several primary isolates of SIV have been identified (Daniel, Letvin et al. 1985; Letvin, Daniel et al. 1985). From these isolates, corresponding molecular clones were isolated including SIVmac251, SIVmac239, and SIV142. Cloned SIVmac239 and SIVmac251 proved to be capable of inducing AIDS-like illnesses in rhesus monkeys *in vivo* (Naidu, Kestler et al. 1988; Kestler, Kodama et al. 1990). Of these two molecular clones, SIVmac239 grows better in rhesus PBMC primary cultures and retains the full-length 41 kD transmembrane protein, which is more like HIV-2. Importantly, SIVmac239 can induce disease symptoms in rhesus monkeys that are remarkably similar to AIDS in humans in a time frame suitable for laboratory investigation. This is a major reason why SIVmac239 is a preferred challenge virus and has been used in HIV-1 vaccine development for many years (Kent, Dale et al. 2001; Evans, Bricker et al. 2005; Kaizu, Weiler et al. 2006; Weiler, Li et al. 2008).

One characteristic of SIVmac239 is that it does not replicate appreciably in rhesus monkey primary alveolar macrophage cultures. However, virus recovered from lung macrophages of a SIVmac239-infected monkey (ID. #316-86) did replicate well in these cultures. This resulted in the isolation of another molecular clone, named SIVmac316, which differs in the *env* sequence by 8 amino acids (6 of them in the gp120 region, 2 of them in the gp41 region) (Mori, Ringler et al. 1992; Mori, Rosenzweig et al. 2000). This clone was associated with the presence of specific pathologic lesions in which infected macrophages were the primary target cells for infection. In addition to a different cellular tropism, the 8 amino acid changes in the *env* sequence caused a significant decrease in dependence on CD4 for entry into cells. This binding difference may explain why the parent strain, SIVmac239, is a pathogenic T cell-tropic virus that is relatively
neutralization-resistant, while SIVmac316 is an R5 macrophage-tropic virus that is less pathogenic \textit{in vivo} and more neutralization sensitive (Puffer, Pohlmann et al. 2002).

Like HIV-1 infected human patients, SIV infected rhesus monkeys typically generate high levels of circulating antibodies against the viral envelope. However, these antibodies have little or no capacity to neutralize typical primary viral isolates \textit{in vitro}, though they often react well with monomeric envelope proteins and envelope-derived peptides (Moore, Cao et al. 1995). Although NAb in rhesus monkeys were not as well studied as the NAb in humans, there have been several key studies in mice and rhesus monkeys. A panel of 17 murine NAb was isolated using a SIVgp120 monomer as an antigen (Kent, Gritz et al. 1991; Kent, Rud et al. 1992). This panel of murine NAb was directed against various regions of SIV gp120 including: 1) the N-terminus; 2) the C1 conserved domain; 3) the V1, V2, and V3 loops; and 4) some conformational epitopes covering the V4 loop. The antibodies were tested for neutralization against SIVmac239 and six variants: SIVmac316, g46, g56, M5, SIV251lab, and SIVΔV1V2 (Johnson, Sanford et al. 2003). Variants g46, g56, and M5 are glycosylation mutants with Asn-to-Gln substitutions in specific N-linked glycosylation sites in the gp120 region. SIV251lab is an uncloned laboratory-adapted SIVmac251 strain. Variant SIVΔV1V2 lacks 100 amino acids within the V1 and V2 loops. Although none of the NAb achieved 50% neutralization against the parent strain SIVmac239 at their lowest dilution (1:20), 12 of the 15 anti-gp120 murine NAb did give 50% neutralization against at least one of the six variants. Nine of them achieved 50% neutralization at dilutions in the order of $10^{-4}$ to $10^{-6}$ against the neutralization sensitive strain SIVmac316 or SIVΔV1V2. These studies provided a starting point for the isolation of 14 fully simian SIV NAb using phage display technology (Glamann, Burton et al. 1998). These Fab fragments were tested against a panel of seven SIV strains including SIVmac239 and SIVmac316 (Johnson, Sanford et al. 2003)
and the pattern of neutralization by these rhesus Fabs was similar to the murine MAbs described above. Again, none of them achieved 50% neutralization against SIVmac239 at its highest concentration. Seven of the Fab fragments were tested against SIVmac316 with 50% neutralizing concentrations ranging from 0.0007 to 0.4 μg/ml. Particularly, Fabs 346-16h and 347-23h showed strong neutralization activity against not only SIVmac316 but also other strains such as M5 and g56 with 50% neutralizing concentrations below 2 μg/ml. Fab 347-23h was about five times more potent than 346-16h against SIVmac316, while 346-16h had broader neutralization activity. Thus, because of their capacity to efficiently neutralize SIV, these two Fabs were selected as potentially good candidates for use in our vaccine study.

4.1.2 A novel recombinant SIV entry inhibitor consisting of a fusion of CD4 and the constant region of rhesus IgG2

Since the discovery of the CD4 molecule as the main receptor facilitating HIV-1 entry into CD4+ T-cells, the concept of using soluble CD4 (sCD4) and CD4-based molecules, such as CD4-IgG2, as competitor molecules to block HIV-1 infection quickly emerged (Kahn, Allan et al. 1990; Schooley, Merigan et al. 1990; Gauduin, Allaway et al. 1996). CD4-IgG2 is a recombinant antibody-like fusion protein wherein both the heavy-chain variable domain and the light-chain variable domain are replaced with the D1D2 domain of CD4 (Allaway, Davis-Bruno et al. 1995). In vitro experiments demonstrated that CD4-IgG2 was more effective than sCD4 in neutralizing primary HIV-1 isolates. Its breadth and potency in neutralizing activity is comparable to IgG1b12 (Trkola, Pomales et al. 1995; Gauduin, Allaway et al. 1996). Both proteins recognize discontinuous binding sites on gp120.
The reason for using subtype IgG2 instead of IgG1 in the CD4-IgG fusion molecule is that the Fc of IgG2 does not bind to the Fc receptor on effector cells as well as the Fc of IgG1. Therefore, it might have less potential to enhance HIV-1 infection or transplacental transmission through Fc-mediated uptake and enhancement of the viral infection (Fust 1997). Fc-mediated enhancement of viral infection is caused by the Fab domain binding the HIV virion, while the Fc domain binds to FcRs on target cells. Since CD4 is the high-affinity receptor for HIV-1/SIV, CD4-IgG2 has the potential to bind and neutralize all strains of the virus and to minimize the potential for the development of resistant HIV-1/SIV strains. In a similar mechanism, due to the lower affinity binding to its complement receptor compared to IgG1, IgG2 also has lower CDC effector function. Taken together, CD4-IgG2 may be an ideal molecule to be used as an immunoprophylactic vaccine candidate. Applying our experimental results with scFv X5 and 3B3hCC, we reasoned that rhesus CD4/D1D2-hinge-C2-C3 subtype 2 (a construct we call RhCD4-Ig2 or N4) (Figure 4.1) may also be a good candidate to test for vaccine efficacy in the rhesus model.

4.2 Materials and methods

4.2.1 Cloning genes encoding 4L6, 5L7, and N4 into rAAV vectors

In earlier work by Desrosier and colleagues, an SIV neutralizing antibody phage display library had been constructed from total RNA extracted from two monkeys infected with SIVmac239 mutants (Johnson, Sanford et al. 2003). From this library, fourteen clones containing SIV neutralizing antibodies were screened and the corresponding Fabs were constructed. Two of these Fab antibodies 346-16h and 347-23h had shown strong neutralizing activity against a group of SIV isolates and were chosen for our study. Construct 4L6 was based on Fab 346-16h and 5L7 was based on Fab 347-23h. rAAV vector plasmids containing SIV scFv entry inhibitors 4L6 and 5L7 were kindly supplied by our collaborator Dr. Bruce Schnepp. The V \text{L} and V \text{H} were joined by
a 15 amino acid glycine-serine (4GS)_3 linker. The antibody variable regions were constructed in the order of V_L-linker-V_H. The two antibody constructs contained a synthetic signal peptide SL1 for optimizing secretion (for details see Chapter 2). After adding the optimized leader sequence, the 4L6 gene is 882 bp while the 5L7 is 855 bp in length. The variable regions were fused to a hCC fragment (687 bp) derived from rhesus IgG2. All DNA coding sequences were synthesized by GeneArt (Regensburg, Germany) using codon-optimized sequences designed for expression in rhesus macaques. The antibody constructs were cloned into an expression cassette containing a truncated CMV promoter similar to the Δ470-173CMV described in Ostedgaard et al. (Ostedgaard, Rokhlina et al. 2005) with a synthetic polyadenylation signal (49 bp) (Levitt, Briggs et al. 1989). This expression cassette was flanked by a modified AAV2 ITR (TRS deleted) to allow for self-complementary rAAV production.

The N4 construct containing the D1D2 domains (623 bp) of rhesus CD4 (synthesized by GeneArt) was also fused to the hCC fragment. This construct maintained the native rhesus CD4 signal sequence. The N4 construct was cloned into an expression cassette containing the full-length CMV enhancer/promoter with the SV40 polyadenylation signal (145 bp), which was flanked by AAV2 ITRs allowing for standard (non self-complementary) rAAV production.

### 4.2.2 Cells and viruses

293K cells (a gift from Dr. Brian Kaspar), a sub-clone of 293T cells, were maintained in 10% CCS/DMEM supplemented with 2.8 μg/ml ciprofloxin. All viruses were made by a modified calcium phosphate DNA plasmid transfection method as described in Chapter 3. The purification process was also the same as reported in Chapter 3 except that the collected supernatant was first concentrated 10-fold by using a MiniKros Pilot KrosFlo TFF machine (Spectrum Labs, Cat.
before iodixanol gradient purification was performed. This improved concentration procedure resulted in high vector recovery. Downstream column purification using an anion exchange resin (HQ Hi-Trap) was performed on an AKTA explorer 100 (GE HealthCare) and vector eluted using a linear NaCl salt gradient. Quality controls consisted of DRP Q-PCR quantitation, endotoxin determination using the limulus amoebocyte lysate (LAL) method, SDS-PAGE purity analysis and sterility testing.

4.2.3 Animals

All experiments involving animals were approved by Nationwide Children's Research Institute Animal Care and Use Committee. The rhesus macaque animal protocol was similar to that described in Chapter 3, but with minor alterations. Rhesus macaques were screened for wild-type AAV1 sero-positivity (Table 4.1: Week 0). Nine animals, which tested either negative at 1:100 serum dilution or had ELISA end point titers of 100, were selected for this experiment. Three rAAV1 vaccine vectors were administered to macaques (n = 3) by intramuscular injection into 4 sites (two sites in each quadriceps, 1.0 ml volume per injection site) at a total dose of 2x10^{13} DRP. Six additional naïve control animals were injected with 4 ml PBS using the same procedure as the experimental animals. Animals were pre-bled, then bled at 2, 4, 6, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, and 55 weeks post-injection. Transgene expression levels in serum were measured by ELISA and all animals challenged with 40 MID_{50} of SIVmac316 by the IV route (a gift from Dr. Ron Desrosiers). Vaccinated animals received the challenge virus 4 weeks post-vaccination. Control animals were challenged in 2 groups (n = 3) 16 week apart.
4.2.4 Enzyme immunoassay

4.2.4.1 ELISA to detect serum levels of 4L6, 5L7, and N4

4L6, 5L7, and N4 levels in serum were measured by ELISA. Briefly, SIVmac239 gp120 (a gift from Dr. Robert Doms, University of Pennsylvania) was used to coat an ELISA plate (100 ng/well) in PBS buffer at 4°C overnight. The next day, each well was blocked with 285 μl 3% BSA in PBST (blocking buffer) for 2 hours with constant shaking at 25°C. All washes were performed 4 times with PBST. Wells were then incubated with 100 μl of diluted sample sera (in PBST) or with standards (4L6, 5L7, or N4) diluted in PBST for 1 hour at 25°C. The secondary antibody (100 μl) was a goat anti-human IgG-Fc HRP conjugate used at a 1:1000 dilution (Bethyl, Cat. #A80-104P, Montgomery, TX) in blocking buffer at 25°C for 30 min. This antibody cross-reacts with rhesus IgG. After washing, the TMB substrate was added (One-Step Ultra TMB-ELISA, Pierce, Cat. #34028) and incubated for 10 min. at 25°C. The reaction was stopped with H₂SO₄ and the plate was read at 450nm. For sample quantification, serum concentrations were obtained by extrapolation from a standard curve generated using software from Molecular Devices (coefficient of linearity ≥ 0.99). The assay sensitivities were calculated to be 0.4 ng/ml for 5L7, 0.6 ng/ml for 4L6, and 4 ng/ml for N4.

Another ELISA method was used to detect antibodies against the Gag core as a means to monitor SIV infection. The method is similar to the one above except that the protein used to coat the plate was SIVsmH4 p55 Gag (100 ng/well) (NIH AIDS Research and Reference Program, Cat. #7748).
4.2.4.2 ELISA to detect anti-idiotypic response against each transgene

To detect antibodies against each transgene (anti-antibody response), another ELISA method was developed. Briefly, 96-well ELISA plates were coated with purified N4, 5L7 or 4L6 in PBS buffer (100 ng/well) overnight at 4°C. Next day, the coating solution was discarded and the plates were blocked with 3% BSA/PBST for 3 hours at 25°C. Each wash was repeated 4 times with PBST buffer. After wash, 100 μl diluted serum samples in PBST were added to the wells and the plate was incubated at 25°C for 1 hour. After another wash, 100 μl of 1:500 diluted mouse anti-human IgG1 Fc specific antibody (Sigma, Cat. #I5385) in the blocking buffer was added to the wells and incubated at 25°C for 30 min. We used the anti-human IgG1 isotype specific secondary antibody to avoid detection of the IgG2 expressed inhibitors (4L6, 5L7, or N4). Finally, the wells were incubated with 100 μl of a 1:1,000 dilution of anti-mouse IgG-Fc HRP conjugate (Sigma, Cat. #A9309) in the blocking buffer for 30 min after wash. The colored substrate reaction was performed with 100 μl of Ultra TMB reagent (refer to Section 4.2.4.1) for 20 min. and the reaction stopped and read as previously described. Samples that had OD450nm values at least two fold higher in antigen-coated wells than that from the non-coated wells were considered positive.

In order to detect cross-reactivity among 4L6, 5L7, and N4 monkey sera to the other antigens, plates were coated with the other two antigens and the same procedures were followed. Positive control wells were coated directly with purified rhesus monkey IgG (Antibodies Incorporated, Davis, CA, Cat. #43-641).
4.2.5 Western strip blot to detect antibodies against the transgenes

The presence of antibodies against the transgenes was further confirmed by a strip western blot using a procedure similar to that detailed in Chapter 2 with minor modifications. Briefly, purified 4L6, 5L7 or N4 proteins were run on a 12% SDS-PAGE gel and the proteins transferred to a PVDF membrane at 100 V for 2 hours at 4°C. Due to high background seen with monkey serum, the blocking buffer was 5% dry milk plus 3% BSA in TBST. The membrane was treated with the blocking buffer for 20 min. at 25°C. Monkey serum samples were diluted at 1:100 in 50% blocking buffer and incubated with the strips overnight at 4°C. Anti-human IgG Fc HRP conjugate antibody (Bethyl, Cat. #A80-104P, Montgomery, TX) was diluted in 50% blocking buffer at 1:250,000 and used as a positive control to confirm the presence of the antigen on the membrane. The strips were washed with TBST and then probed with a mouse anti-human IgG1 antibody (Sigma, Cat. #15385) at a 1:5,000 dilution in 50% blocking buffer for 1 hour at 25°C. The strips were washed again and incubated with a goat anti-mouse IgG HRP conjugated antibody (Sigma, Cat. #A9309) at a 1:10,000 dilution in 25% blocking buffer for 30 min. Finally, the ECL Plus Western kit was used for immunodetection as previously described.

4.2.6 Viral loads

Viral loads in monkey plasma were measured by quantitative real-time RT-QPCR at Bayer Reference Testing Laboratory (Emeryville, Calif.), as previously described (Cline, Bess et al. 2005). Briefly, virus was sedimented from 1.0 ml of undiluted plasma by centrifugation in a micro-centrifuge at 4°C. Supernatant was removed and the pellet was re-suspended in 50 μl of proteinase K digestion buffer (3 M Guanidinium HCl, 50 mM TrisCl, pH 7.6, 1 mM CaCl₂) containing 1 mg/ml proteinase K) and the tube then incubated at 37°C for 1 hour. After digestion, 200 μl of RNA carrier and 250 μl of isopropanol were added to pellet RNA. After a 10 min.
centrifugation, the supernatant was removed and the pellet was washed in 70% ethanol and air-dried. RNA was dissolved in 30 μl DNase and RNase free water containing 1 mM DTT and 1 unit/μl RNAsOUT (Invitrogen, Carlsbad, CA). cDNA was made using RT and random hexamer primers in a thermocycler. Immediately following reverse transcription, a mixture of amplification primers, probe, and Taq polymerase was added to the reaction tube. The assembled reaction was run on an ABI 7700 Sequence Detection System (Applied Biosystems). Copy numbers were calculated by interpolation from a standard curve of RNA standards using ABI system software.

4.2.7 SIV neutralization assay

Neutralization was measured by a secreted alkaline phosphatase (SEAP) reporter assay under the control of a *tat*-responsive promoter (in the LTR). These experiments were performed as previously described (Means, Greenough et al. 1997). Similar to the TZM-Bi cell line used in HIV neutralization, a human CD4+ CEMx174 cell line containing the SIV LTR-SEAP construct (called CEMx174 LTR-SEAP) was used in this neutralization assay. Basal levels of SEAP activity from this cell line are low and infection with SIV results in a dramatic increase in SEAP expression within 48 to 72 hours. The SEAP levels directly correlate with the amount of infecting virus. Since SEAP is secreted into the cell media, chemiluminescent measurement of SEAP activity in the cell free supernatant supplies a rapid, sensitive, and quantitative assay for SIV infectivity and neutralization. 50% neutralization titers of the vaccinated monkey sera against SIVmac316 stock were determined using this assay.
4.2.8 Statistical analysis

Significance for the null hypothesis (no protection) was performed using a two-tailed Fisher’s exact test method (Walker 2002) by using numbers of infected or protected monkeys from each individual or combined group. A p value was calculated from a web-based computer program (http://www.langsrud.com/fisher.htm) for each case. Normally distributed student’s t-test was used to compare the viral loads between control and vaccinated monkeys. A p value was calculated from a web based computer program (http://www.physics.csbsju.edu/stats/t-test.html) for each week between control and vaccinated groups. A p value less than 0.05 was considered statistically significant and a p value less than 0.01 was considered highly significant.

4.3 Results

4.3.1 Construction of the three SIV entry inhibitor vectors

Based on the experimental results from the previous Chapters, we showed that scFv antibody fusion with an hCC fragment was superior to scFv antibody or whole antibody (IgG), with respect to serum half-life, and ultimately steady-state serum concentration. We extended this result to a general formula for our vaccine constructs: anti-viral construct + hCC. In addition to minimize transgene immunogenicity, we made gene transfer vectors expressing simian proteins, just as one might use all human sequences for a human vaccine application. The only exception is a 15 amino acid (4GS)₃ linker region, which is artificial (Das, Kriangkum et al. 2004). We constructed three IgG-like entry inhibitors (2 scFv and a CD4-based molecule) from two potent neutralizing Fabs and a truncated rhesus CD4 molecule containing the two N-terminal domains (D1D2) (Figure 4.1, details in the materials and methods) (Johnson, Sanford et al. 2003). The Fab molecules were shown to neutralize several isolates of SIV including SIVmac316, thereby giving us the opportunity to test their neutralizing activities after rAAV mediated gene transfer to
macaques. As described earlier, CD4-IgG has a potential to be used as an immunoprophylactic agent. From the published Fab and CD4 D1D2 domain sequences, we codon-optimized the variable heavy chains, variable light chains, and CD4 D1D2 domains for maximal expression in rhesus macaques. Using a similar strategy as we employed for scFv 3B3hCC, each pair of variable light and variable heavy chains was connected by the linker and fused to the rhesus IgG2 constant region (hCC) to generate 4L6 and 5L7. The 4L6 clone was from the sequence of the Fab clone 346-16h and the 5L7 was from the sequence of Fab clone 347-23h. Due to their small gene sizes, 4L6 and 5L7 were incorporated into SC vectors with a shortened CMV (ΔCMV) promoter and a synthetic polyadenylation signal. RhCD4-IgG2 (i.e., N4) contains the first two domains of rhesus CD4 linked to rhesus IgG2 hCC. N4 was incorporated into a regular SS rAAV vector with the standard CMV promoter and SV40 polyA signal because this construct was made before the sCMV based SC vector was available to us. Each construct was sequenced and the expression (and secretion) of the transgene was confirmed using HeLa and 293 transfection studies by ELISA (data not shown). Using the above three constructs, the corresponding rAAV1 viruses were made by transient DNA plasmid transfection of 293K cells using the calcium phosphate method described in Chapter 2. Virus purification was performed using iodixanol gradients and FPLC using single-use anion exchange HQ resins (refer to the materials and methods).

4.3.2 In vivo expression levels in NHPs from the three vectors

Our experience with animals receiving the 3B3hCC vaccine suggested that perhaps AAV pre-existing immunity played a role in someway limiting transgene expression. Therefore, we chose to pre-screen our animals for evidence of previous wild-type exposure using an AAV1 binding ELISA. Nine animals with a serum titer of ≤ 1:100 were selected for this study (Table 4.1). The three vaccine vectors were administered to macaques (n = 3) as a single intramuscular injection into 4 sites at a total dose of 2x10^{13} DRP (two sites per leg). Sera from the animals were then
analyzed over the ensuing year for the presence of the transgene expression, as well as, anti-idiotypic responses against the transgene products. Transgene expression levels in serum were measured by the SIV gp120 ELISA.

Overall, expression was very robust (Figure 4.2), with the 4L6 group having the highest expression on average over the course of the experiment. All three monkeys from this group possessed ≥ 100 μg/ml by week 4. More importantly, the expression continued to increase and peak between weeks 16 - 20 at 300 to 425 μg/ml. Levels then plateaued between 250 to 300 μg/ml over the next 12 months (Figure 4.2: 4L6). In the 5L7 group, the three monkeys appeared to behave differently. Monkeys C053 and C004 expressed high levels of 5L7 within the first two weeks, compared to monkey C002, whose level remained low. Interestingly, monkey C004 then dropped to almost to zero at week 4, while monkey C053 continued to increase. Monkey C002 maintained a nearly linear increase of 5L7 expression within the first 24 weeks, with peak levels (264 μg/ml) reached at week 24, which then plateaued to ~ 250 μg/ml (Figures 4.2). Both monkeys C053 and C004 showed huge increases in gp120 binding activity after week 6, which suggested SIV infection after challenge (monkeys were challenged at week 4).

The three monkeys vaccinated with N4 showed yet another picture. All three monkeys (C079, D043, D172) had lower transgene expression ranging from 1.7 to 15 μg/ml during the 55 weeks, which was an order of magnitude lower than that seen in the 4L6 and 5L7 groups. Monkey C079 had a rapid increase of N4 expression that peaked at week 6. Its expression levels were sustained through week 55. Monkeys D043 and D172 both had similar low level expression at week 2. D172 rose after week 12 and reached similar levels as those observed in monkey C079. Like animals C053 and C004 in the 5L7 group, the apparent large increase of N4 binding antibodies in
monkey D043 (as well as six-naïve-control monkeys) after week 6 indicated SIV infection after challenge. In conclusion, all vaccinated animals except C004 had persistent expression over 55 weeks. Importantly, 5 out of 6 monkeys in the 4L6 and 5L7 groups had expression levels in the expected range for therapeutic levels after week 4.

4.3.3 Challenge studies with the three injected and control groups

In previous experiments, the expression of scFv X5 peaked at 4 weeks post-injection, and then dropped significantly afterwards. Similarly, 3B3hCC injected monkeys had high expression levels at week 2, but expression dropped in 3 out of 4 monkeys by week 4. Considering these experimental results and the very good serum expression data at weeks 2 and 4, we decided to challenge the vaccinated monkeys and three naïve control monkeys with 40 MID<sub>50</sub> of SIVmac316 at week 4 post-vaccination by the IV route. To satisfy the statistical power analysis, three more naïve control monkeys were challenged in the exact same manner and at the same virus dose 16 weeks later.

To initially monitor SIV infection following challenge, we performed a Gag (SIVsmH4 p55) binding ELISA on serum samples to detect elicitation of an anti-gag humoral response. SIVsmH4 gag shares ~ 87% similarity to SIVmac316 gag and, therefore, polyclonal responses are expected to be readily detected (Hirsch, Adger-Johnson et al. 1997; Johnson, Sanford et al. 2003). As Figure 4.3 shows, there were no Gag responses at weeks 8 and 12 post-vaccination in the 4L6 group, while two monkeys (C004 and C053) in the 5L7 group showed positive responses to Gag during these time points. In spite of low-level N4 expression, a single animal (D043) showed positive Gag antibody responses at week 12. As expected, all 6 naïve control monkeys showed robust anti-Gag responses indicative of an active SIV infection. These results agree with the
dramatic increase seen in the 3 presumably infected animals (C004, C053, and D043) with regards to their gp120 binding ELISA (Figure 4.2).

Infection in these animals and naïve controls was confirmed by quantitative viral load analysis showing replicating virus in their sera (Figure 4.4). In contrast, no viral load was detected at all weeks measured to date (51 weeks) in any of the monkeys in the 4L6 group, one monkey (C002) in the 5L7 group, and two monkeys (C097 and D172) in the N4 group. Therefore, it appears sterilizing immunity was provided to these animals by gene transfer. Importantly all six naïve control monkeys had high viremia by week 2 - 4 post challenge, with peak plasma viral loads ranging between 6.5 to 8 log\(_{10}\) RNA copies/ml. Only one control animal had viremia below 5 log\(_{10}\) RNA copies/ml in the post-acute phase of the infection. The three vaccinated and infected monkeys had peak viral loads below 6.5 log\(_{10}\) RNA copies/ml. After peak viral loads at week 4, monkey C004 had its viral load plateau to around 5 log\(_{10}\) RNA copies/ml, monkey C053 plateaued to around 2.7 log\(_{10}\) RNA copies/ml, and monkey D043 plateaued to around 4 log\(_{10}\) RNA copies/ml. In addition, monkey D043 showed a delayed viremia by two weeks compared to the other infected monkeys. From these results, we concluded that sterilizing immunity was achieved in six out of nine vaccinated monkeys. Delayed viremia was observed in one infected monkey in the N4 group (D043).

The plateau in the viral load (i.e., set point) following acute infection has been shown to be a predictor for eventual disease progression (Horton and De Rosa 2007). Here, viral loads reached peak levels at week 4 and then stabilized between 8 to 51 weeks post-challenge, with week 12 values representing median values for almost all infected monkeys. Therefore, viral load at week 12 was taken as the set point. The average set point for six naïve control monkeys was 6.0 ± 0.97
log_{10} RNA copies /ml. Compared to this average, the viral set point in monkey C053 was reduced by 2.5 log_{10} RNA copies/ml, which translates to a 3,000 fold viral load reduction (RNA copies/ml). The set point in monkey D043 was reduced by 1.7 log_{10} RNA copies/ml (50 fold reduction) and that in monkey C004 was reduced from 1.4 to 4.3 log_{10} RNA copies/ml (25 fold reduction). Therefore, these three monkeys had a 90 to > 99% reduction in viral set points compared to the average set point of the naïves, which was statistically significant (student t-test, p < 0.05). As a consequence of the different viral set points, four of the six naïve control macaques had to be euthanized between 57 and 60 weeks after infection due to AIDS-related illnesses. On the contrary, at the time of this writing, all of the nine vaccinated animals are still alive without AIDS-related illnesses for 60 weeks. Thus, the expressed inhibitors may continue to reduce plasma viremia in the three infected macaques.

During the acute-phase (weeks 2, 4, and 8), analysis of viral loads also revealed a significant difference between the three infected monkeys and the naïve-control monkeys (student t-test, p < 0.05) (Table 4.3). In addition, during the early chronic phase infection (weeks 12, 16, and 24), the viral loads were also significantly lower than that from the naïve monkeys (student t-test, p < 0.05). However, no statistical differences were observed for later weeks (student t-test, p > 0.05). The gradually increasing p values over time may be partially explained by the decreasing viral load in naïve animal D016, which was not observed in the other 5 naïves. Of interest, this monkey had the lowest binding antibodies at both acute and chronic phases that remained stable during the chronic phase for all weeks measured (Figure 4.2). One difference between this monkey and the other five naïve animals is the presence of the MHC haplotype Mamu A01 allele (Table 4.1). The A01 allele has been demonstrated to be associated with a potent SIV cytotoxic T lymphocyte epitope that exhibits strong control of SIV replication in both vaccinated (DNA-Gag prime-Ad5
boost) and naïve-control monkeys challenged with SIVmac239 (Casimiro, Wang et al. 2005). In the vaccinated group, one Mamu A01 monkey was C004, which showed a complete loss of gp120 binding activity at the time of challenge at week 4 and was then infected. However, we did not observe a similar trend of gradual loss of viremia at later weeks in this monkey. Monkeys D014 (N4) and D172 (4L6) also possess the A01 allele but were not infected. So, if we only compare the viral loads between monkeys without the potent control for this haplotype, the three vaccinated and infected monkeys had significantly lower viral loads at all weeks measured compared to the five non-A01 allele naïve control monkeys (student t-test, p < 0.05) (Table 4.4). Furthermore, the differences in six out of 10 weeks measured were highly significant (student t-test, p < 0.01) (Table 4.4), which was not observed in the comparison between the vaccinated group and six-naïve-control monkeys (Table 4.3). Again, the most likely explanation for the significantly lower viral loads compared to controls is that a significant amount of the challenge virus was blocked by the expressed inhibitors at the initial stage of the infection. From these results, we concluded that significantly lower viral loads were achieved in the vaccinated but infected monkeys compared to the five-naïve-control non-A01 allele monkeys.

Due to the small sample sizes, a two-tailed Fisher’s exact test was used to evaluate the significance of the vaccine’s efficacy. The numbers of either infected or protected monkeys in each vaccine and naïve groups were listed in a 2x2 contingency tables (Table 4.5). Comparison between vaccinated and control monkeys (Table 4.5) yielded a p value = 0.03 and indicates that a significant difference was achieved between vaccinated monkeys compared non-vaccinated controls. Furthermore, with death as an endpoint, the vaccine recipients (0/9) showed a highly significant difference to unvaccinated controls (4/6) (p = 0.01) (Table 4.6). Dividing animals into groups according to vaccine, the p-values were calculated as 0.01, 0.08, and 0.3 for 4L6, N4, and
5L7, respectively (Table 4.5). Therefore, only inhibitor 4L6 provided statistically significant protection with these small numbers. Clearly, larger dose cohorts may prove that the other molecules can also provide a benefit.

4.3.4 In vitro serum neutralizing activity

As predicted by the above inhibitor serum levels, all of the nine vaccinated monkeys had serum neutralizing activity against SIVmac316 after vaccination (Table 4.7). Similar to the viral load data, there were clear differences among the three individual vaccine groups. In the 4L6 group, the animals achieved high levels of serum neutralizing activity that agreed well with their high transgene expression levels. Neutralizing end point titers ranged from 1:640 to 1:5,120 at week 2 through week 12. In the 5L7 group, all three monkeys achieved high levels of serum neutralizing activity at week 2. Consistent with the binding data, monkey C004 then lost detectable neutralizing activity at week 4 (day of challenge), while animal C002 demonstrated an increase in levels, again consistent with serum transgene levels. Monkey C053 presented another scenario, where the serum neutralizing titer was the highest on the day of challenge (1:20,480), but surprisingly, this animal still became infected. In the N4 group, all three monkeys achieved similar neutralizing activities up until 12 weeks post-vaccination. Consistent with the serum N4 inhibitor levels (Figure 4.2: N4), serum neutralization titers were 1 - 2 orders of magnitude lower than that from the 4L6 and 5L7 groups. The high titer of neutralizing activity observed in D043 at week 12 was a result of challenge virus infection. All 3 animals from this group had low NAb titers (1:64) at the time of challenge yet only D043 became infected. This suggests that this titer (with this molecule) may represent a minimal threshold level where either protection or infection will occur.
4.3.5 Anti-antibody responses in the infected animals

We next explored possible causes for the failure to protect against SIV infection in the three vaccinated animals. The first clue came from monkey C004 (5L7 group), who had high levels of 5L7 antibody at week 2 but then rapidly declined (both in levels and NAb activity) (Figure 4.2 and Table 4.7). We characterized the anti-transgene antibody response by ELISA, using purified 5L7 as the solid phase antigen. It became clear that this animal had a very strong anti-5L7 antibody response to the 5L7 antigen starting at week 3 (Figures 4.5 and 4.6). This result suggested that the expressed 5L7 antigen elicited high-levels of anti-5L7 antibodies that neutralized its activity based on binding and in vitro neutralization assays resulting ultimately in this animal becoming infected.

Additional anti-transgene ELISA results using the corresponding expressed antigen showed that all three SIV infected monkeys (C004, C053, D043) produced anti-antibody responses, with monkey C004 having the most robust response, by far. A detectable anti-5L7 was seen in C053 response starting at week 6, while the N4 animal D043 had an anti-N4 response that became detectable at week 4. All three animals has a peak response at week 8, that gradually decreased to base line levels by 32 weeks for C053 and D043 and 44 weeks for C004 (Figure 4.6). Intriguingly, none of the six protected monkeys showed a detectable anti-transgene response. To confirm the ELISA results, western strip blot analysis was performed using C004 and C053 animal sera (5L7 animals). As shown in Figure 4.7, monkey C004 sera contained high concentrations of anti-5L7 antibodies at 2 - 12 weeks, which waned dramatically after week 32 (Figure 4.6). In contrast, C053 produced approximately 50 to 100 fold less anti-5L7 antibody than C004 estimated by western band intensity and ELISA signals for the two monkeys (Figure 4.5 and Figure 4.7).
A question regarding the timing of challenge (week 4) and detectable anti-transgene activity arises with animals C053 and D043. Both of these animals did not have detectable anti-transgene antibodies and had *in vitro* neutralization activity at the time of challenge, but still became infected. While purely speculative, low level anti-antibody concentrations may have been present that effectively competed with the expressed inhibitor, resulting in breakthrough. The low level (1:64) of neutralizing activity in the N4 animal D043 is consistent with such a scenario, while this becomes more problematic with C053 due to its high level NAb activity at time of challenge.

To gain additional insight into what were the targets of the anti-transgene antibodies, we performed additional ELISA assays to look at cross-reactivity between different antigens. To that end, we coated plates with the different antigens (5L7, 4L6, or N4) but used serum samples from the other groups against the antigens. The serum samples incubated with their own antigen served as positive controls. Since all three animals showed peak anti-transgene antibody responses at week 8, we only used this time point for this experiment. Serum from naïve control monkey C009 (week 0) was used as the assay negative control. As Figure 4.8 shows, anti-transgene antibodies from each animal had a unique cross-reactivity profile. Anti-5L7 antibodies from monkey C004 also cross-reacted with 4L6 but not with N4. This suggested that these antibodies were raised against the variable and/or framework sequences in the scFv domains 5L7 that also recognized those in the 4L6 molecule. This result may not be unexpected, considering the sequence similarities between 4L6 and 5L7. Their protein sequences have 85.4% homology. Furthermore, some regions such as FR1, FR3, and FR4 in 4L6 share up to 95% homology with 5L7 (Figure 4.9). Therefore, the observed anti-antibody response for monkey C004 was most likely an anti-idiotypic response.
Anti-antibodies against 5L7 from monkey C053 reacted with all three inhibitors at very similar levels, which suggested that the common regions among 5L7, 4L6, and N4 (i.e., hCC portion of IgG2) might be the immunogenic target in this monkey. The Fc domain in our scFv fusion antibody may possess unique epitopes since the CH1 of IgG2 was omitted. The linker region of the inhibitor is also a foreign sequence that is potentially immunogenic, but is not present in N4 and, therefore, this is not likely a target. These data provide another possible mechanism for infection in this monkey, namely, blockage of Fc effector functions. Consistent with this supposition, it was recently demonstrated by Burton and colleagues that a functional Fc fragment is essential for in vivo neutralization through ADCC effector functions for NAb IgG1b12 (Hessell, Hangartner et al. 2007).

Anti-antibodies against N4 from monkey D043 did not cross-react with either 4L6 or 5L7. This result suggests that the anti-antibodies were most likely generated against the D1D2 domains. Similar to the mechanism in monkey C004, anti-D1D2 antibodies might block the function of N4 molecule, resulting in infection. A difference between these two antibodies was their relative concentrations. In monkey D043, the N4 level at week 4 might be at a threshold for infection, where only a small amount of anti-D1D2 antibody could tip the balance from protection to infection without significantly affecting the measurement of N4 in serum.

4.4 Discussion

4.4.1 Why was protection achieved this time?

The goal of our studies was to demonstrate the proof-of-concept that antibody gene transfer using a rAAV platform could generate circulating HIV-1/SIV neutralizing activity high enough to achieve protective immunity. Our data showed that biologically-active, rhesus-derived scFv
antibodies and an rhesus CD4 fusion with the constant region of IgG2 were efficiently expressed following intramuscular injection of rAAV1 vectors. More importantly, after a single administration, sterilizing immunity was achieved in six out of nine vaccinated monkeys challenged with a virulent dose of SIVmac316. Using a two-tailed Fisher’s exact test, the rAAV1 vaccine achieved significant protection (p < 0.05), comparing vaccinated and control groups. Accordingly, the vaccine recipients showed a highly significant survival rate compared to unvaccinated monkeys (p = 0.01). However, breaking the data into vaccine groups, only vaccine 4L6 provided significant protection (Table 4.5). For the vaccinated, but infected monkeys, benefits such as reduction in viral set points and viral loads, delayed viremia (only in D043), living healthy and longer were also obtained compared to naïve control animals.

Several important experimental changes were incorporated into this challenge study. First, animals were pre-screened for low or absent pre-existing binding antibodies against AAV1 (Table, 4.1). Secondly, we exploited the ability to select and clone naturally occurring rhesus-derived antibody Fabs with broad specificity and potent reactivity against several SIVmac clones (Johnson, Sanford et al. 2003). Studies on the mechanism of IgG recycling suggested that the half-lives of rhesus-derived proteins should be longer when expressed in rhesus monkeys (Ober, Martinez et al. 2004; Lencer and Blumberg 2005). This may also reduce the likelihood of a host immune response compared to use of human proteins (scFv X5 and 3B3hCC). Thirdly, not only the injection dose (2x10^{13} DRP/monkey) was lower than that of our previous injections (3.5x10^{13} DRP/monkey), but also more injection sites (four) were used to help minimize vector immunogenicity and the possibility of overloading myocyte secretion and protein folding pathways (Rutkowski and Kaufman 2004). Lastly, incorporation of SC vectors appears to have a significant effect on the observed levels in the NHPs that mirrored what we observed in the pilot
rodent studies. Due to these improvements, sustained serum levels of the NAbs in the range of 40 - 200 μg/ml were observed for five out of six animals receiving rAAV1/ΔCMV.4L6 and rAAV1/ΔCMV.5L7, at the day of challenge. The N4 animals showed significantly lower serum levels (3 - 10 μg/ml) at that time, which may be related to the use of a standard SS rAAV1 and its own natural leader sequence. Another possible reason for the low level of N4 could be due to the short half-life of sCD4 (45 minutes after IV injection in human) (Allaway, Davis-Bruno et al. 1995).

Serum IgG is regulated by the neonatal MHC-class-I-like Fcγ receptor FcRn. IgG is spared from lysosomal degradation by binding to FcRn in endosomes at mildly acidic pH and is released at a slightly basic pH after moving back to the cell surface (Hinton, Xiong et al. 2005). The receptor FcRn is widely expressed in many different cells and the recycling mechanism is thought to be constitutive (Rodewald and Kraehenbuhl 1984; Roberts, Guenthert et al. 1990). Two mutants of IgG2 (M428L and T250Q) have been reported to increase pH-dependent binding to FcRn (Hinton, Moustafa et al. 2004). These mutants had a 2.5-fold longer half-life than wt IgG2 in primates and may be possible targets for future studies.

Use of an IV challenge route instead of the intra-rectal route may also have contributed to our success in blocking challenge virus infection. Challenge via the IV route allows the inhibitor antibody and inoculum virus to interact directly. The rectum has a single protective layer of columnar epithelium overlying tissue rich in activated lymphoid cells. The infection and protection mechanisms through this membrane are not well studied (Cranage, Sharpe et al. 2008). At this point, we do not know whether the inhibitor levels we achieved in this study would prevent viral infection if we challenged via the rectum. Intra-rectal challenge usually requires
higher concentrations of challenge virus, and so it is unclear what serum levels of inhibitors would be required to achieve sufficient local membrane concentrations to block challenge virus infection. In any regard, more comparison studies are needed to test whether our approach works with intra-rectal challenge.

Combining all of the above enhancements, we demonstrated that six out of nine vaccinated animals were completely protected against a virulent virus challenge, while all six naive control animals became infected. Importantly, this represented statistically significant protection using our antibody gene transfer paradigm. Specifically, all three animals in the 4L6 group were completely protected, which was most likely due to the high levels of 4L6 present at the day of challenge (100 – 200 μg/ml). Monkey C002 in the 5L7 group was also completely protected while it had only ~ 40 μg/ml of 5L7 at the day of challenge. Monkey C004 was infected due to the apparent low concentration of 5L7 at the day of challenge, which appeared due to an anti-antibody response against 5L7 (Figure 4.5, 4.7). Although monkey C053 had a very high 5L7 concentration at the time of challenge (170 μg/ml), it still became infected. The mechanism for this infection may be related to the production of anti-antibodies against the Fc portion of the 5L7 molecule.

In the case of N4, the concept of using D1D2 domains of sCD4 to block HIV-1/SIV infection appeared to be particularly effective even at the low serum levels observed. Monkey D043 with an intermediate expression level of N4 (~ 5.0 μg/ml) was infected, while monkey D172 with a slightly lower concentration was protected. This result implies that the threshold for the challenge virus infection may be around 5.0 μg/ml for N4. A more detailed dose-response study is needed to determine the actual threshold. Anti-D1D2 antibodies produced in this monkey (D043) are
most likely responsible for the infection. Other factors such as genetic background and/or receptor CD4/co-receptor CCR5 expression levels in individual monkeys may also influence the end result and actual thresholds in a particular animal. Although D043 was infected in this group, the viremia was delayed by two weeks. Considering the half-life of SIV in plasma is only about three to four minutes (Zhang, Dailey et al. 1999) and the life-cycle of the virus is approximately 24 hours (Firpo, Axberg et al. 1992), the lack of detectable viremia in this monkey in the first two weeks suggests a low level of initial infection. At the same time, there might be partial inhibition between newly produced SIV particles and the expressed inhibitor N4. This result agrees with the finding of a significantly lower viral set point than the control naïve monkeys. Therefore, the expressed N4 not only effectively blocked the initial infection in two monkeys but may have also been beneficial for the infected monkey by delaying the burst of viremia for two weeks and, consequently, lowering the viral set point.

Although we did not define the lowest level of circulating 4L6 required to achieve sterilizing immunity against SIVmac316 in this challenge model, we do know that monkey C002 was completely protected with only $40 \mu g/ml$. The threshold for complete protection in 4L6- and 5L7-injected monkeys may fall within the range of $50 - 200 \mu g/ml$ as predicted by passive immunization studies (Mascola, Louder et al. 1997; Mascola, Lewis et al. 1999; Shibata, Igarashi et al. 1999; Mascola, Stiegler et al. 2000; Mascola, Lewis et al. 2003). In sharp contrast, two of the monkeys in the N4 group achieved sterilizing immunity and one had delayed viremia at inhibitor levels ranging from 3 to $10 \mu g/ml$. This raises the question of why did animals require much lower levels of N4 to achieve benefits as compared to the levels of circulating 4L6 and 5L7? One reason might be the differences in the mechanisms of neutralization between 4L6, 5L7 and N4. The N4 molecule, originated from the natural receptor molecule, binds trimeric gp120
directly and may induce cross-linking among envelope spikes (Zhu, Olson et al. 2001). The N4 cross-linked gp120 complex may then be cleared by ADCC effector functions of IgG2 (Jacobson, Lowy et al. 2000). Therefore, it seems that a single SIV virus particle may only need one N4 molecule to neutralize, while every viral gp120 spike needs to be occupied by at least one NAb molecule such as 4L6 or 5L7 in order to achieve a successful blockade of the challenge virus (Yang, Kurteva et al. 2005). Given the fact that a virus has about 72 gp120 spikes on the surface, this would allow approximately 72-fold lower threshold to achieve protection for the N4 molecule as compared to 4L6 or 5L7 (Gelderblom, Hausmann et al. 1987). Therefore, for non-antibody inhibitors such as what we developed here, the required concentration for a therapeutic effect may be much lower than that determined from passive immunization studies mentioned above. Considering the importance of effector function of the Fc part, a fusion of D1D2 domains with the constant region of IgG1 might be even more potent and may require even lower concentrations to achieve a benefit since IgG1 displays high antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) effects. Therefore, a comparison between CD4-IgG1 and CD4-IgG2 seems worth testing \textit{in vivo} in the future.

Due to the effectiveness of N4 at very low serum concentration as compared to 4L6 and 5L7, it clearly warrants further study including: 1) mutagenesis or protein evolution could be performed on this molecule to increase its serum half-life; and 2) use of a SC vector with inclusion of a synthetic leader peptide (SL1) that may further increase serum N4 concentration. It may not be too difficult to achieve high efficacy against multiple primary isolates with modified N4 molecules and vectors since CD4 binding is very conserved in HIV-1 (Saha, Zhang et al. 2001). Considering the fact that almost all challenge virus doses used in NHP studies far exceed the infectious inoculums in the real world for HIV infection (in order to achieve 100% or near 100%...
infection rate in control animals) (Ma, Abel et al. 2004; McDermott, Mitchen et al. 2004; Vlasak
and Ruprecht 2006), we may not need extremely high concentrations of the N4 molecule for
humans to be protected. The highest transmission rate for HIV infection is ~ 90% through blood
transfusion (Donegan, Stuart et al. 1990). Other estimated infection rates are even lower
(http://www.who.int/inf-pr-2000/en/pr2000-25.html). The infection rate during childbirth is only
25% in the developing world and 1 to 2% in the USA. Infection rate through needle-sharing is
only 0.67% per act. Therefore, we may not need extremely high concentrations of the N4
molecule to block the HIV-1 infection.

4.4.2 Possible causes for anti-antibody responses in the three infected monkeys

Of the nine vaccinated monkeys, monkey C004 in the 5L7 group was observed to produce a large
amount of anti-idiotypic antibodies, while monkeys C053 and D043 produced relatively small
amounts of anti-antibodies (Figure 4.5).

Of the three molecules delivered to the monkeys, the N4 molecule was theoretically least likely to
generate a humoral immune response because the D1D2 and hCC domains were both derived
from rhesus sequences. These sequences are highly conserved among non-human primates. The
small amount of anti-N4 antibody observed in monkey D043 may be directed against a new
epitope generated in the region between the hinge and D1D2. The conformational change at the
beginning of Fc region of the N4 molecule, due to the omission of CH1, might be another source.

The Fabs of 4L6 and 5L7 were generated from different individual monkeys, and, therefore, anti-
idiotypic responses are possible and likely to arise in specific animals based on haplotype
(Johnson, Sanford et al. 2003). Again, the production of anti-idiotypic response can be explained
by Jerne’s network hypothesis (refer to Chapter 1 and 2). Monkey C004 produced anti-5L7 antibodies (Ab2) upon expression of 5L7 (Ab1), Ab1 were measured in our gp120 ELISA and Ab2 were detected in our 5L7 ELISA and western strip blot (Figure 4.5, 4.7). According to the continued high expression levels of 4L6, 5L7, and N4 in these un-infected monkeys (Figure 4.3), it is very likely that the vaccinated but infected monkeys continue to express these inhibitors. The apparent low expression levels measured in monkey C004 between weeks 4 and 8 could be partly due to Ab2 binding to the serum 5L7, which interfered with the determination of the actual 5L7 concentration in the gp120 ELISA. This phenomenon was also observed for monkey M1 in rAAV1/CAG.3B3hCC-injected monkeys (refer to Chapter 3). More detailed experiments are needed to test this hypothesis. On the other hand, monkey C053 produced anti-antibody response against Fc region of 5L7. These anti-antibodies are predicted not to interfere with either the gp120 binding assay for measuring its concentration nor in vitro neutralization assay for its neutralizing activity. In fact, monkey C053 had the highest serum level and highest neutralization titer at the day of challenge. These data suggest that Fc effector functions might be very important for in vivo neutralizing activity. Our data appear to confirm and extend the recent work of Hessell et al., in which a dramatic decrease in the ability of IgG1b12 to protect macaques against SHIV challenge when Fc receptor binding site was mutated (Hessell, Hangartner et al. 2007).

4.4.3 Future studies

Herein, with the SIV molecular constructs in our monkey challenge model, we used a dose of $2 \times 10^{13}$ DRP/monkey. Based on this, we predict that we would need about $2 \times 10^{14}$ DRP for an average 70 kg adult. Clearly, to vaccinate large numbers of individuals, such dosing is beyond foreseeable production capabilities. Therefore, additional studies are needed to define what level of dose reduction is achievable that still provide significant protection. Specifically, what is the lowest inhibitor concentration needed for sterilizing immunity using improved molecules with
increased potency at lower doses? We note that synergistic and additive effects were observed among several neutralizing human NAbs administered in combination in passive immunization studies (Li, Baba et al. 1997; Mascola, Louder et al. 1997). Synergistic interactions may allow lower concentrations of each individual NAb to act effectively to achieve sterilizing immunity.

As numerous publications have shown, a single NAb that neutralizes all subtypes does not exist (Montefiori and Evans 1999; Kwong, Doyle et al. 2002; Warren 2002; Binley, Wrin et al. 2004; Burton, Desrosiers et al. 2004; Burton, Stanfield et al. 2005). At present, almost all challenge studies including ours have used a single cloned or primary HIV-1/SIV isolate (Dittmer, Stahl-Hennig et al. 1995; Kent, Dale et al. 2001; Warren 2002; Johnston, Johnson et al. 2005; Quinnan, Yu et al. 2005). Adding several NAb genes together in a single vaccine cocktail might expand the breadth and potency against primary viruses from multiple clades.

It would be of interest to repeat the 3B3hCC studies using a SC vector with an IV challenge and pre-screening the animals to see if protection is now afforded. In Chapter 3, we constructed several rAAV vectors which can express 3B3hCC. This may lay a foundation for future human clinical trials to test 3B3hCC-like molecules. Accordingly, the International Aids Vaccine Initiative (IAVI) is exploring the use of rAAV1/b12hCC, based on the results of this study. A rAAV1 SC vector will be generated and used in a Phase 1 dose escalation trial in the United States to determine: 1) safety; 2) use of AAV1 as a delivery system in normal adults; 3) duration of antibody expression; and 4) serum-neutralization activity against several primary HIV-1 isolates.
4.4.4 Combining with T cell vaccines

In addition to NAbs preventing HIV-1 infection, HIV-1-specific CTLs can act to control viral replication by removing infected cells. Although the Merck T cell vaccine recently failed in the STEP clinical trial, it remains under intense study for the potential to protect against low-dose challenge or, possibly, to augment with a NAb based vaccine (Catanzaro, Roederer et al. 2007; Martinon, Brochard et al. 2008). Clearly, a combination of NAbs with T cell activity would represent the ideal situation by providing time for the host to reactivate HIV-specific memory T-cells that could clear or modify the clinical course of HIV-1 infection should infection still occur. An additional benefit would be a lowering of the HIV-1 viral load that may also reduce the rate of transmission (Quinn, Wawer et al. 2000), resulting in fewer infections that would be predicted to impact the global pandemic based on currently accepted mathematical models (Blower, Schwartz et al. 2003).

4.4.5 Conclusions

As a proof-of-concept experiment, we demonstrated the feasibility of using rAAV-mediated gene transfer into muscle tissue to achieve sufficient levels of circulating, neutralizing antibodies to block virus infection in six out of nine rhesus monkeys. In addition, we achieved significantly lower viral loads and set points for those vaccinated but infected monkeys, compared to naïve control monkeys. For one of the infected monkeys, we also observed delayed viremia by two weeks. Considering the diversity of HIV-1, our approach will be limited by the breadth and potency of the NAbs and inhibitors. With the recent identification of patient sera showing broad and potent HIV-1 neutralization activity superior to IgG1b12 (Li, Migueles et al. 2007), additional molecules will likely follow. In addition, this novel approach can be applied not only
as a prophylactic HIV vaccine, but also as a therapeutic vaccine against other infectious diseases and possibly cancers (Galun, Terrault et al. 2007).
<table>
<thead>
<tr>
<th>Vector</th>
<th>Animal#</th>
<th>MHC Status</th>
<th>0</th>
<th>8</th>
<th>20</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>4L6</td>
<td>05C020</td>
<td>A02, B01</td>
<td>&lt;100</td>
<td>4000</td>
<td>50,000</td>
<td>20,000</td>
</tr>
<tr>
<td></td>
<td>05C066</td>
<td>A08, B01, B08</td>
<td>&lt;100</td>
<td>20,000</td>
<td>20,000</td>
<td>10,000</td>
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<td></td>
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<td>A01</td>
<td>&lt;100</td>
<td>5000</td>
<td>10,000</td>
<td>5000</td>
</tr>
<tr>
<td>5L7</td>
<td>05C002</td>
<td>A02, A08, B01</td>
<td>100</td>
<td>20,000</td>
<td>200,000</td>
<td>100,000</td>
</tr>
<tr>
<td></td>
<td>05C004</td>
<td>A01, A02</td>
<td>100</td>
<td>100,000</td>
<td>40,000</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>05C053</td>
<td>B08</td>
<td>100</td>
<td>80,000</td>
<td>50,000</td>
<td>1000</td>
</tr>
<tr>
<td>N4</td>
<td>05C079</td>
<td>A02</td>
<td>&lt;100</td>
<td>160,000</td>
<td>50,000</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>05D043</td>
<td>none</td>
<td>100</td>
<td>200,000</td>
<td>50,000</td>
<td>10,000</td>
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<tr>
<td></td>
<td>05D172</td>
<td>A01</td>
<td>100</td>
<td>180,000</td>
<td>50,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Naive Control</td>
<td>05C009</td>
<td>A08, B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>05D016</td>
<td>A01, A02, B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>05D225</td>
<td>A02, B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>05C016</td>
<td>A02, A08, B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>05D059</td>
<td>B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>05D152</td>
<td>B01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not done.
Table 4.2: Neutralizing activity of a panel of the rhesus anti-SIV Fab (the two potent and broad Fabs were marked in red frames) (Johnson, Sanford et al. 2003)

<table>
<thead>
<tr>
<th>Fab</th>
<th>Con. of Fab fragment (µg/ml) reducing infectivity by 50%&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIV239</td>
</tr>
<tr>
<td>346-4</td>
<td>–</td>
</tr>
<tr>
<td>346-16h</td>
<td>–</td>
</tr>
<tr>
<td>346-19h</td>
<td>–</td>
</tr>
<tr>
<td>346-23h</td>
<td>–</td>
</tr>
<tr>
<td>346-24h</td>
<td>–</td>
</tr>
<tr>
<td>346-25h</td>
<td>–</td>
</tr>
<tr>
<td>347-1h</td>
<td>–</td>
</tr>
<tr>
<td>347-2h</td>
<td>–</td>
</tr>
<tr>
<td>347-5h</td>
<td>–</td>
</tr>
<tr>
<td>347-6h</td>
<td>–</td>
</tr>
<tr>
<td>34710h</td>
<td>–</td>
</tr>
<tr>
<td>347-19h</td>
<td>–</td>
</tr>
<tr>
<td>347-23h</td>
<td>–</td>
</tr>
<tr>
<td>B6</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Concentration of Fab fragment required to reduce infectivity of the indicated virus by 50%. –, 50% neutralization was not achieved even at the highest concentration tested. ND: Not determined.
Table 4.3: Student’s t-test on viral loads between the vaccinated but infected monkeys and all six naïve control monkeys

<table>
<thead>
<tr>
<th>Week</th>
<th>P-value</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>32</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>&lt;0.020</td>
<td>0.026</td>
<td>0.033</td>
<td>0.019</td>
<td>0.033</td>
<td>0.067</td>
<td>0.045</td>
<td>0.056</td>
<td>0.085</td>
<td>0.110</td>
</tr>
</tbody>
</table>

Table 4.4: Student’s t-test on viral loads between the vaccinated but infected monkeys and the five naïve non-A01 allele control monkeys

<table>
<thead>
<tr>
<th>Week</th>
<th>P-value</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>32</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>&lt;0.035</td>
<td>0.007</td>
<td>0.032</td>
<td>0.005</td>
<td>0.002</td>
<td>0.004</td>
<td>0.009</td>
<td>0.008</td>
<td>0.012</td>
<td>0.020</td>
</tr>
</tbody>
</table>
### Table 4.5: Fisher’s exact test for the vaccinated monkeys (infected or blocked)

<table>
<thead>
<tr>
<th>Compare groups</th>
<th>Vaccinated</th>
<th>Number of monkeys blocked</th>
<th>Number of monkeys infected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All vaccinated vs. naïve controls</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>6</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
<td>P = 0.03</td>
<td></td>
</tr>
<tr>
<td>4L6 vaccinated vs. naïve controls</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
<td>P = 0.01</td>
<td></td>
</tr>
<tr>
<td>5L7 vaccinated vs. naïve controls</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
<td>P = 0.33</td>
<td></td>
</tr>
<tr>
<td>N4 vaccinated vs. naïve controls</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
<td>P = 0.08</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4.6: Fisher’s exact test for all the vaccinated monkeys (alive or dead)

<table>
<thead>
<tr>
<th></th>
<th>Number of monkeys dead</th>
<th>Number of monkeys alive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All vaccinated ones</td>
<td>0</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Totals</td>
<td>4</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
<td>P = 0.01</td>
</tr>
</tbody>
</table>
Table 4.7: Neutralization activity of monkey sera at different weeks without pre-heating

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Animals</th>
<th>Infection (+/-)</th>
<th>Week 0</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>C009</td>
<td>+</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>20480</td>
</tr>
<tr>
<td></td>
<td>D016</td>
<td>+</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>20480</td>
</tr>
<tr>
<td></td>
<td>D225</td>
<td>+</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>&lt;32</td>
<td>81920</td>
</tr>
<tr>
<td>4L6</td>
<td>C020</td>
<td>-</td>
<td>&lt;32</td>
<td>640</td>
<td>2560</td>
<td>2560</td>
<td>5120</td>
</tr>
<tr>
<td></td>
<td>C066</td>
<td>-</td>
<td>&lt;32</td>
<td>5120</td>
<td>5120</td>
<td>2560</td>
<td>5120</td>
</tr>
<tr>
<td></td>
<td>D014</td>
<td>-</td>
<td>&lt;32</td>
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<td>2560</td>
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<td>-</td>
<td>&lt;32</td>
<td>480</td>
<td>1280</td>
<td>2560</td>
<td>5120</td>
</tr>
<tr>
<td></td>
<td>C004</td>
<td>+</td>
<td>&lt;32</td>
<td>3840</td>
<td>&lt;32</td>
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<td>40960</td>
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<td></td>
<td>C053</td>
<td>+</td>
<td>&lt;32</td>
<td>1920</td>
<td>20480</td>
<td>30720</td>
<td>40960</td>
</tr>
<tr>
<td>N4</td>
<td>C079</td>
<td>-</td>
<td>&lt;32</td>
<td>128</td>
<td>128</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>D043</td>
<td>+</td>
<td>&lt;32</td>
<td>64</td>
<td>128</td>
<td>64</td>
<td>81920</td>
</tr>
<tr>
<td></td>
<td>D172</td>
<td>-</td>
<td>&lt;32</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

Note: Values are the serum dilution at which relative SEAP activities were reduced 50% compared to virus control wells. The lowest dilution tested was 1:32.
Figure 4.1: Model structures of the Ig-like constructs of 4L6, 5L7 (V\textsubscript{L}-l-V\textsubscript{H}-Ig2), and N4 (i.e., RhCD4-Ig2). In the constructs, scFv fragments (for 4L6 and 5L7) and D1D2 domains of CD4 (for N4) were linked with rhesus IgG2-derived hinge-CH2-CH3 (hCC).
Figure 4.2: Entry inhibitor concentrations in monkeys following rAAV vaccination. The data were grouped according to the rAAV injected. The Y-scales in the graphs are different due to their different production levels. The arrows and dotted lines indicate the day of challenge (DOC). The concentrations for the three-naïve-control animals were calculated according to the 5L7 standards and represent part of the humoral immune response against the challenge virus. In the naïve control group, three monkeys were challenged at week 4 post vaccination; three monkeys were challenged at week 16 post vaccination.
Figure 4.3: SIV Gag ELISA to detect SIV infection. OD450nm ELISA values (see materials and methods) are plotted versus the serum samples from monkeys at weeks 4, 8, and 12 (w04, w08, w12) post vaccination. All the sera were diluted at 1:100 in PBST. A value of 3.0 at OD450nm indicated that the reaction was saturated. All samples were tested on a 96-well ELISA plate coated with SIVsmH4p55gag protein.
**Figure 4.4: Viral loads after challenge.** Plasma SIV genome copy numbers (log_{10}/ml) from monkeys in the three immunized and the naïve control groups (see materials and methods for viral load measurement) are plotted versus weeks after challenge. Three naïve control animals were challenged with vaccinated monkeys at same time and three were challenged 16 weeks later.
Figure 4.5: Anti-antibody response detected by ELISA. All sera were diluted at 1:100 in buffer PBST. An OD450nm value of 3.0 indicates saturation of the color reaction in ELISA. RhIgG is a positive control, in which commercial Rh IgG protein was coated directly on the ELISA plate (30 ng/well). Serum from naïve control monkey C009 before vaccination served as the negative controls for all plates. The red dashed line indicates ELISA detection sensitivity. Only the samples have OD450nm values above this line were considered positive.
Figure 4.6: Anti-antibody response in monkey C004 detected by ELISA. All sera were diluted at 1:1000 in buffer PBST. An OD450nm value of 3.0 indicates saturation of the color reaction in ELISA. RhIgG(+) is a positive control, in which commercial Rh IgG protein was coated directly on the ELISA plate (30 ng/well). Serum from monkey C004 before vaccination served as a negative control.
Figure 4.7: Western strip blot of anti-antibody response against 5L7. All sera from the indicated monkeys and times (labeled as week numbers post immunization) were diluted at 1:100 in the blocking buffer (5% dry milk plus 3% BSA in TBST). The samples were incubated at 4°C overnight in individual strips in 50% of the blocking buffer. The serum from control monkey C009 before vaccination was used as a negative control (lane: -). The strip directly incubated with anti-human IgG HRP conjugate at 1:250,000 dilution served as a positive control (lane: +).
Figure 4.8: Anti-antibody cross-reactivity among N4, 4L6, and 5L7 (ELISA). Serum samples at week 8 were diluted 1:100 in buffer PBST, and incubated with 5L7, 4L6, or N4 coated plates as indicated by the protein name. Serum from monkey C009 served as a negative control. Monkey sera incubated with their own antigen served as positive controls. The red dashed line indicates ELISA detection sensitivity. Only the samples have OD450nm values above this line were considered positive.
Figure 4.9: DNA sequence comparison between scFvs 4L6 and 5L7. The top strand is the 4L6 sequence and the bottom is the 5L7 sequence. FR1, FR2, FR3, and FR4 are framework regions. CDR1, CDR2, and CDR3 are complementary-determining regions. A high degree (85.4%) of sequence identity between 4L6 and 5L7 in the V<sub>H</sub> domains suggests a shared target for the anti-antibody response in monkey C004.
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