SUBTYPE-A AND SUBTYPE-B HIV-1 P24 BASED FIV VACCINES: EFFICACY AND MECHANISMS OF CROSS-PROTECTION

By

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To my loving family and dear friends; through their love and support, I was able to pursue my dreams

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HIV:	Human Immunodeficiency Virus
HIV-1_{UCD1}:	HIV subtype-B strain
HIV-1_{N20}:	HIV subtype-A strain
$HIV1_{LAV}$:	HIV subtype-B strain
FIV:	Feline Immunodeficiency Virus
FIV_{Bang}:	FIV-Bangston (subtype-A *gag-pol-*$env_{v1\text{-}v2}$, B $env_{v3\text{-}v9}$)
FIV_{Pet}:	FIV-Petaluma (subtype-A)
FIV_{FC1}:	FIV-FC1 (subtype-B)
Fel-O-Vax:	Commercial FIV vaccine, Fort Dodge Animal Health, Fort Dodge, Iowa.
IWV:	Inactivated whole virus
SPF:	Specific pathogen free cats
LTNP:	Long term non-progressor
KK10:	HIV epitope associated with LTNPs
Gag:	Group specific antigen
Pol:	Polymerase
Env:	Envelope
Vif:	Viral Infectivity Factor
Vpu:	Viral Protein U
Vpr:	Viral Protein R
Nef:	Negative Regulatory Factor
Tat:	Trans-Activator of Transcription
Rev:	Regulator of Viral Protein
RT:	FIV Reverse Transcriptase
PR:	Protease

PRIs:	Protease inhibitors
IN:	Integrase
DU:	Deoxyuridine triphosphate
HAART:	Highly active antiretroviral therapy
ART:	Anti-retroviral therapy
MA:	Matrix
CA:	Capsid
NC:	Nucleocapsid
HVTN:	HIV Vaccine Trials Network
Orf2:	Open Reading Frame 2
LANL:	Los Alamos National Laboratories
NRTI:	Nucleoside Reverse Transcriptase Inhibitor
NNRTI:	Non-nucleoside Reverse Transcriptase Inhibitors
PI:	Protease Inhibitor
FI:	Fusion Inhibitors
VNA:	Virus-neutralizing antibody
SC:	Vaccinated subcutaneously
RID:	Radial immunodiffusion
FeT-J:	Feline T-cell line developed By J.K. Yamamoto
EU:	Endotoxin
TBV:	Total blood volume
SFU:	Spot forming units
ELISpot:	Enzyme-linked immunosorbent spot
pQE30:	Expression vector is based on the T5 promoter transcription-translation system

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Published data shows HIV-1_{UCD1} p24 based vaccine protects cats from FIV infection. Understanding the mechanism of vaccine protection could provide a blueprint to the development of an HIV-1 vaccine for humans. The studies were performed by evaluating the protective ability of the vaccine-induced antibody response in three separate passive-transfer studies. The first involving vaccine antiserum, the second involving ammonium sulfate purified antibodies, and the third using antibodies purified using ammonium sulfate precipitation and caprylic acid fractionation. The data suggested that the protective efficacy of HIV-1_{UCD1} p24 (subtype-B) based vaccine was not mediated by vaccine-induced antibody immunity but most likely mediated by vaccine-induced cellular immunity. In addition protective efficiency varied between cats vaccinated with subtype-B HIV-1_{UCD1} p24 (67% protection) and subtype-A HIV-1_{N20} p24 (25% protection). In addition, IFNγ ELISpot analysis using separate peptide pools of overlapping 15mers of FIV and HIV-1 p24 showed cross reactivity between FIV peptides and HIV-1 p24-vaccinated cat sera and between HIV-1 peptides and sera from either FIV p24-vaccination a Fel-O-Vax FIV-vaccinated cats. Some vaccinated cats reacted to peptides corresponding to the KK10 HIV-1 epitiope in humans, which is associated with long term non-progressor (LTNP) HIV-1 infected individuals.

CHAPTER 1
INTRODUCTION

Background

Human Immunodeficiency (HIV) and feline immunodeficiency virus (FIV) are members of the lentiviral family Retroviradae [1],[2]. These viruses are characterized by their ability to infect terminally differentiated, non-dividing cells such as macrophages; by their genomic organization of *group specific antigen* (*gag*), *polymerase* (*pol*), and *envelope* (*env*) in addition to regulatory genes (Figure 1-1); and by their utilization of the enzyme reverse transcriptase (RT) to synthesize DNA using their RNA genome as a template [1]. The error-prone nature of RT accounts for the high mutation rate observed among these viruses [2].

Human Immunodeficiency Virus

HIV-1 infection was first reported in 1983 as a retroviral infection of humans [3]. HIV-1 is the etiological agent of acquired immune deficiency syndrome (AIDS) which causes a progressive deterioration of the host immune system, characterized by the loss of $CD4^+$ T-helper lymphocytes. The CD4 receptor is the primary receptor of viral entry into the cell. Other major receptors utilized by the virus include CCR5 and CXCR4 [4]. Its genome contains three major genes, consisting of *gag, pol*, and *env*. The primary p160 mRNA transcript of HIV-1 is translated into Gag and Pol proteins. Gag p55 is further cleaved into p24 core protein, p17 matrix protein, p9 nucleocapsid, and proline-rich p6 protein involved in viral assembly. Pol precursor protein is cleaved RT, protease, and integrase [5]. The HIV-1 genome also encodes two regulatory proteins, Tat and Rev, which regulate transcription and viral RNA transport, respectively. The virus also contains four accessory genes, *vif, vpu, vpr* and *nef*, which are implicated in viral pathogenesis [6]. The accessory and regulatory genes are described in detail below.

HIV-1 Viral Infectivity Factor (Vif)

Vif-like proteins are associated with all known lentiviruses with the exception of equine infectious anemia virus (EIAV) [7]. Vif appears to be needed at the late stage of viral replication and may suppress antiviral activity of T lymphocytes and macrophages, which are the main cells infected in humans and cats [8,9]. Macrophages also serve as viral reservoirs of infection even after the acute stage of infection. It is believed that Vif is important for the retention of viral infectivity because it acts as an inhibiter to the antiviral pathway involving APOBEC3G, a messenger RNA editing enzyme [10]. Vif may prevent the editing of the early single strand product of reverse transcription. It is also believed that this activity results in the prevention of many mutations, which could compromise the key structural proteins, regulatory proteins, and enzymes and as a result, aids in retaining viral infectivity.

HIV-1 Viral Protein U (Vpu)

Vpu protein promotes the degredation of the CD4 antigen in the host cell, and this prevents the binding of CD4 molecules to viral gp 120 in the infected cells [11]. Vpu also causes the formation of ion channels in the surface membrane of the infected cells. The ability of Vpu to form these channels seems to correlate with the cell's ability to release the virus [12].

HIV-1 Viral Protein R (Vpr)

Vpr protein appears to be involved in viral pathogenesis and is important in the infection of macrophages [13]. Vpr is also involved, to a lesser extent in the infection of other cells. This protein causes host cell division to stop at the G2 stage, and induces the apoptosis of infected cells. Vpr acts as a shuttle protein from the cytoplasm to the nucleus by facilitating the transfer of the pre-interaction complex through the nuclear pores.

HIV-1 Negative Regulatory Factor (Nef)

Nef protein is synthesized very early in HIV infection and promote viral fitness through multiple mechanisms [14]. First, in order to prevent super-infection of the infected cells, Nef causes the incorporation and destruction of surface CD4 in the lysosomes. Second, this protein causes the down regulation of the major histocompatability complex-I (MHC-I) expression, which affects antigen presentation, and thereby reduces the destruction of infected cells by cytotoxic T lymphocytes. Third, Nef increases viral infectivity by inducing the infected macrophages to secrete MIP-1 alpha and MIP-1 beta. These chemokines have a chemotaxic effect on resting $CD4^+$ T lymphocytes, causing the migration of the cells to the site of infection. This process in turn increases the chance for these cells to become infected, since free virions do not retain infectivity very long when circulating in the blood [7,15].

HIV-1 Trans-Activator of Transcription (Tat)

Tat protein is a spliced gene product of 14 kD in size [2].Tat protein interacts with *tat responsive element,* an RNA loop structure on the 3' end of the viral long terminal repeat (LTR), to upregulate HIV gene expression [16].

HIV-1 Regulator of Viral Protein (Rev)

Rev protein is 19 kD and is the product of doubly spliced mRNA. Rev protein is involved in the regulation of viral RNA expression [2].

HIV-1 Classification

There are two types of HIV presently known to exist: HIV-1 and HIV-2 [Figures 1-1 and 1-2] [17]. Based on the genetic sequences, HIV-1 has been classified into three groups: the M (major) group, O (outlier) group, and the N (non-outlier) group. HIV-1 M group is further divided into nine subtypes (A-D, F-H, J, and K) [18]. The majority of HIV infections worldwide

are the result of M group viruses [18]. There is also an increasing amount of infection caused by inter- and intra-subtype recombinants [19].

HIV-1 Treatment

When HIV-1 was first identified over two decades ago, there were no effective treatments available. There were also few treatments for the secondary opportunistic infections, which are associated with HIV-1 infection. Presently there are several drug options for HIV-1-positive individuals. A number of drugs first approved for HIV-1 therapy were nucleoside analogs, inhibitors of RT activity. Subsequent classes of drugs included protease inhibitors and non-nucleoside RT inhibitors followed by more recent drugs shown in Table 1-1. The use of two or more of these drugs together was initially called highly active antiretroviral therapy (HAART) and more recently renamed as anti-retroviral therapy (ART) [20]. ART has been very effective in prolonging the life expectancy of infected individuals and can reduce the amount of circulating virus to nearly undetectable levels. During drug induced remission, the virus may still remain dormant in viral reservoirs within the host, such as the testes, brain, and retina [21]. Financial constraint has been the major limiting factor for these drugs to have a major global impact. With most of the HIV-infected individuals residing in developing countries, a desirable strategy to arrest the viral spread would be by the development of an effective prophylactic vaccine.

Feline Immunodeficiency Virus

FIV is a lentivirus of domestic cats, which causes an immunodeficiency syndrome strikingly similar to HIV infection in humans [22]. This similarity between these two viruses extends to the genome organization of *5'- LTR-gag-pol-env-LTR-3'* and the presence of select regulatory genes (Figure 1-1) [9].

The level of FIV infection of domestic cats worldwide has been estimated to be between 1% to 28% [23]. FIV variants have also been classified into five subtypes A-E [23]. FIV

infection usually results in $CD4^+$ T lymphocyte reduction [24]. Similar to HIV strains, the virulence of FIV strains varies [23]. Like HIV-infected individuals, there is also the presence of long term non-progressor (LTNP) subjects among FIV-infected cats [23]. The LTNP cats remain disease free for a period of time, which is well over the mean disease onset of acute and moderate progressors.

FIV has a broad tropism by infecting both $CD4^+$ and $CD8^+$ T lymphocytes, B cells, circulating monocytes, and monocytes of the CNS [25]. The tropism of the virus is determined by the viral envelope characteristics. FIV has the same genomic organization as most other lentiviruses with coding sequences of the *gag, pol,* and *env,* in addition to accessory genes flanked by open reading frames (ORFs) [7].

FIV Group Associated Antigen (Gag)

The Gag polyprotein is approximately 50 kD and is produced by translation of unspliced viral genomic RNA transcript. This polyprotein is cleaved by viral protease, into three proteins matrix (MA, 15kD), capsid (CA, 24 kD), and nucleocapsid (NC, 7 kD) [7]. The importance of including Gag proteins into a potential HIV vaccine is now recognized throughout the HIV vaccine community. The current trials in the HIV Vaccine Trials Network (HVTN) all incorporate *gag* gene or Gag protein (Table 1-2). The presence of many conserved epitopes coupled with the strong immune response stimulated by this protein support its use as a vaccine immunogen [26]. Many T-cell epitopes are located within the Gag region. These epitopes would be important to facilitate the removal of infected cells in order to stem the spread of the infection.

FIV Envelope (Env)

FIV Env is the product of a singly spliced 4.4 kD strand of mRNA [7], [27,28]. The Env glycoprotein precursor is composed of SU (95 kD) and TM 39 (kD), and these two glycoproteins are assembled on the viral membrane as non-covalently bonded surface glycoprotein. As is the

case with HIV, FIV SU is the most divergent of the viral proteins [9], and mutations of this glycoprotein can affect the tropism of the virus.

FIV Polymerase (Pol)

The *pol* gene region of the ORF encodes a polyprotein, which is cleaved by FIV protease (PR) into four proteins: PR, RT, integrase (IN), and deoxyuridine triphosphate (DU) [7]. The cleavage order of these proteins affect the number of infectious virions produced [29].

FIV Protease (PR)

Protease is a viral enzyme, which processes the Gag and Gag-Pol polyproteins to the final viral components. Comparison between FIV and HIV-1 PRs have revealed common amino acid (aa) recognition sites, facilitating the utilization of experimental protease inhibitors against both lentiviruses [30]. However, the majority of commercial HIV-1 protease inhibitors (PRIs) do not work against FIV PR.

FIV Reverse Transcriptase (RT)

FIV RT is composed of a 61kD polypeptide and is the product of cleavage of the Gag-Pol polyprotein by FIV protease [7]. There is a significant sequence homology between HIV-1 RT and FIV RT, and FIV RT is also sensitive to the nucleoside analogs utilized in the treatment of HIV-1 [31]. FIV RT may also undergo mutations, which result in resistance to these drugs [31].

FIV Deoxyuridine Triphosphate (DU)

FIV DU is a protein trimer consisting of 14.3kD subunits [32]. DU exists in both eukaryotic and prokaryotic cells, facilitating the hydrolysis of dUTP to dUMP to prevent the miss-incorporation of deoxyuridine during the synthesis of DNA. Low levels of DU in the infected non-dividing cells can reduce FIV replication. The virus overcomes this obstacle by producing its own DU and thereby enhancing its fitness [7].

FIV Integrase (IN)

FIV IN is roughly 32kD and is derived from the C-terminus of the precursor Pol protein [7]. The C-terminus of IN is important to the interaction of the enzyme with DNA. It is responsible for site-specific cleavage of the ends of FIV DNA, and for the transfer and disintegration of DNA strands [33].

FIV Viral Infectivity Factor (Vif)

Vif is produced from spliced RNA about 5.2 kB long [7]. *Vif* gene is found in all lentiviruses except EIAV. The size and genome location are also well conserved. This gene affects the infectivity of the virions by mechanisms thought to be similar to those of HIV-1 [34].

FIV Open Reading Frame 2 (Orf2)

FIV Orf2 is the transactivating factor of FIV [7] [35]. It is a 9.3 kD protein product of multiply spliced strands of mRNA. Orf2 contains an N-terminus cystene-rich region but lacks the basic domains which are essential for HIV-1 Tat activity. Recent studies suggest that Orf-2 may also serve as Vpr [36].

FIV Regulator of Viral Protein (Rev)

Rev protein facilitates the triggering of late gene expression in the virus life cycle [37]. It promotes nuclear export of mRNAs enabling their translation. Similar to HIV-1, FIV Rev is mediated by the *rev responsive element,* which is located on the 3' end of target mRNAs. Rev is involved in nuclear localization and the transfer of genetic material in and out of the nucleus.

HIV/FIV Vaccine Model

FIV is similar to HIV in morphology, genetic sequence, and pathogenesis [9]. In light of this, vaccine approaches, which are effective against one of these viruses, are likely to provide valuable insights into the other. Both HIV and FIV have common challenges of high mutation rate due to the error prone nature of viral RT, inter- and intra-subtype recombinations, and the

need to generate broad vaccine protection against a range of different subtypes [38]. Cytotoxic T lymphocytes (CTL) responses to HIV and FIV are mediated by $CD8^+$ Tcells, which target epitopes on the whole virus. Several of these epitopes target have been identified on Gag and Env [23]. The period of time until the onset of clinical symptoms may vary between strains and between subjects. There is however a significantly greater numbers of LTNP cats infected with FIV. This may be due to the fact that HIV is believed to only have been discovered in the human population for about two decades, while FIV has evolved with its host much longer in evolutionary time [5,9]. HIV Gag protein appears to be less divergent phylogenetically than FIV and simian immunodeficiency virus (SIV) (Figure 2). The epitopic characters of the attenuated strains may well provide insights into the ideal targets for vaccine design. Currently the average survival time for infected individuals has increased considerably causing the CDC to launch new studies to recalculate this figure [39].

In 1993 at University of California at Davis, researchers in the School of Veterinary Medicine were able to elicit a protective immune response in 96% of cats immunized with whole-cell or cell-free FIV vaccines and subsequently challenged with heterologous FIV [40]. This was the first instance of an effective vaccine formulation against an AIDS lentivirus. The exact nature of the protective response was not certain. The next step was to determine the mechanism of the protective response. The protection observed initially appeared to correlate with a strong humoral response, including in the generation of virus neutralizing antibodies(VNAs) [41]. However, the VNA titer of the study animals did not correlate completely with protection.

One of the first viral proteins to be recognized by the host immune system is the core p24. There are also several CTL epitopes found on core p24 [42]. This protein is the structural

component of the viral capsid and is relatively conserved compared to the viral Env. HIV-1 and FIV p24 have 31% aa identity and 63% aa homology based on length adjusted analysis. The identity observed with p24 was much higher than those for SU, TM, and MA. Therefore, it was a good target for a vaccine immunogen to explore the concept of evolutionarily conserved epitopes among lentiviruses. The use of HIV-1 p24 as a vaccine immunogen facilitated the formation of cross-reactive antibodies to FIV (Figure 1-3), despite having only 63% homology. Thus, the possible utility of HIV-1 p24 protein as vaccine immunogen against FIV was noted, and this led to the following evaluations.

Rationale and Goals for the Proposed Studies

Following immunization with a subtype-B HIV-1_{UCD-1} p24 vaccine, 82% of specific pathogen free (SPF) cats were able to elicit a successful protective immune response to challenge with FIV [38]. The 31% aa identity between HIV-1 and FIV p24 sequences in combination with the cross-protective ability of the HIV-1 p24 vaccine suggested that there may be some protective epitopes within the regions of homology, and these may be evolutionarily conserved between the two lentiviruses. Based on these observations, our hypothesis is that some of these epitopes conserved among the lentiviruses may serve as potential vaccine immunogens against FIV. This hypothesis will be tested by performing the studies for the following specific aims: 1) determine if humoral immunity is responsible for the protection conferred by HIV-1_{UCD1} p24 vaccine against FIV and 2) determine if cross-protection against FIV is conserved among different subtypes of HIV-1.

Specific Aims

Determine if humoral immunity is responsible for the protection conferred by HIV-1UCD1 p24 vaccine against FIV: Cross-reactive antibodies to HIV-1 proteins were generated in cats infected with FIV [38](Figure 3). This implied that there were some common epitopes

conserved between both viruses despite their evolution in different host species. The strongest area of cross reactivity observed by HIV-1 whole-virus western blot was core p24 protein. Hence the role that HIV-1 p24 antibodies play in cross-protection against FIV was evaluated. The following two experimental approaches were undertaken to achieve this goal.

A. B-cell mapping of cross-reactive antibodies to FIV p24.
B. Protective efficacy of the antibodies from FIV p24-vaccinated cats was determined in vivo by passive-transfer studies against FIV challenge.

In order to identify the cross-reactive B-cell epitopes, sera from HIV-1 p24-vaccinated cats were analyzed by FIV p24 peptide-based ELISA. The substrates for the ELISA consisted of 28-30mer peptides that spanned FIV p24 with 11-13 aa overlaps. The published B-cell and T-cell epitopes available from Los Alamos National Laboratories (LANL) were compared to those on the vaccine p24 antigen and the challenge virus. Since the p24 capsid of the virus is not expressed on its surface such as its Env, it is unlikely that it would elicit a protective B-cell response. However, this arm of the immune system could be used to identify T-cell epitopes, such as T-helper epitopes for B-cell responses and possibly for CTL activity.

Determine if p24 from subtype-A HIV-1 with a different epitope repertoire can be utilized as a vaccine antigen: The hypothesis proposed for this dissertation is that some of the p24 epitopes conserved among HIV-1 subtypes may work together as a vaccine immunogen against FIV. Whereas, the central hypothesis of our laboratory is that some of the cross-protective HIV-1epitopes, prevalent among viral structural and enzymatic proteins, against FIV should be useful as vaccine epitopes against HIV-1 in humans. As of yet, there has been no studies demonstrating the epitopes found on HIV-1 p24 proteins are all protective against FIV. For this reason, p24 from subtype-A HIV-1 will be used to test our immediate hypothesis by identifying the protective p24 epitopes, which are conserved among subtypes A and B of HIV-1.

Furthermore, the studies proposed for this dissertation are the first step towards answering the central hypothesis of our laboratory. Hence, the effort to identify cross-protective HIV-1 epitopes against FIV may provide a blueprint for the development of HIV-1/AIDS vaccines against HIV-1 subtypes prevalent worldwide. Overall, our studies will not only benefit the development of second-generation FIV vaccines for veterinary medicine but will also contribute to the development of a HIV-1 vaccine for human medicine.

Table 1-1. Current drugs available to treat HIV infection [a]

Drug Class	Drug Name	Drug Mechanism
Nucleoside Reverse Transcriptase Inhibitor (NRTI)	Lamivudine (3TC), Zidovudine (AZT), Zalcitabine (ddC), Stavudine (d4T), Abacavir (ABC), Tenofovir (TDF) Didanosine (ddI)	Nucleotide or nucleoside analogues lacking a hydroxyl group at the 3' end.
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)	Efavirenz (EFV), Nevirapine (NVP), Delaviridine (DLV)	Binds to HIV-1 RT and alters the active site to reduce nucleotide binding.
Protease Inhibitor (PI)	Nelfinavir (NFV), Amprenavir (APV), Fosamprenavir (FPV), Saquinavir (SQV), Lopinavir (LPV/r), Ritonavir (RTV),Atazanavir (ATZ), Indinavir (IDV)Tipranavir (TPV)	Mimics Gag-Pol cleavage site and competes with protease, causing the production of immature non-infectious virions.
Fusion Inhibitors (FI)	Enfuvirtide (EFV)	Binds to gp41 and blocks the fusion of the virus and cellular membrane.
CCR5 Antagonists	Maraviroc (FDA approved April 24, 2007)	Blocks binding to CCR5

[a] Adapted from Sierra S. et al.2005 and updated with newly approved anti-HIV drugs [43].

Table 2-2. Current HIV vaccine trials

Phase / Sub-type	Producer	Product Name	Prime Formulation Constituents									
			Gag	Pol	Env	Nef	Tat	Rev	Vpr	Vpu	PR	RT
I/B	Sanofi Pasteaur	ALVAC vCP1452	+		+	+					+	+
I/B	Chiron	Gag and Env DNA/PLG Microparticles	+		+							
I/B	Merck	MRKAd5 HIV-1 Gag	+									
I/B	Therion	TBC-M358 TBC-M335										
I/B	Wyeth	GENEVAX gag-2962	+	+	+	+	+	+				
I/B	Wyeth	GENEVAX gag-2962	+									
I/B	Pharmexa-Epimmune	EP-1043 + EP HIV-1090	+	+	+	+			+	+		
I/B	GeoVax	HIVB DNA pGA2/JS7 #2	+		+		+	+		+	+	+
I/B[a]	NIH VRC	VRC-ADV-014/ VRC-ADV-009	+	+	+							
I/C	AlphaVax	AVX-201	+	+	+	+						
I[b]	Pharmexa-Epimmune	EP HIV-1233	+	+	+	+		+	+			
I/B	UP[c]	PENNVAX-B	+	+	+							
I/B	Merck	MRKAd5	+	+		+						
I/A	NIH VRC	VRC-HIVDNA-044			+							
II/B[b]	NIH VRC	VRC-HIVDNA-016	+	+	+	+						
II/B	Merck	MRKAd5 Trivalent	+	+		+						
II/B	Merck	MRKAd5 Trivalent	+	+		+						

[a] Also contains clade A,B,C *env.*
[b] Includes multiple specific epitopes.
[c] University of Pennsylvania.

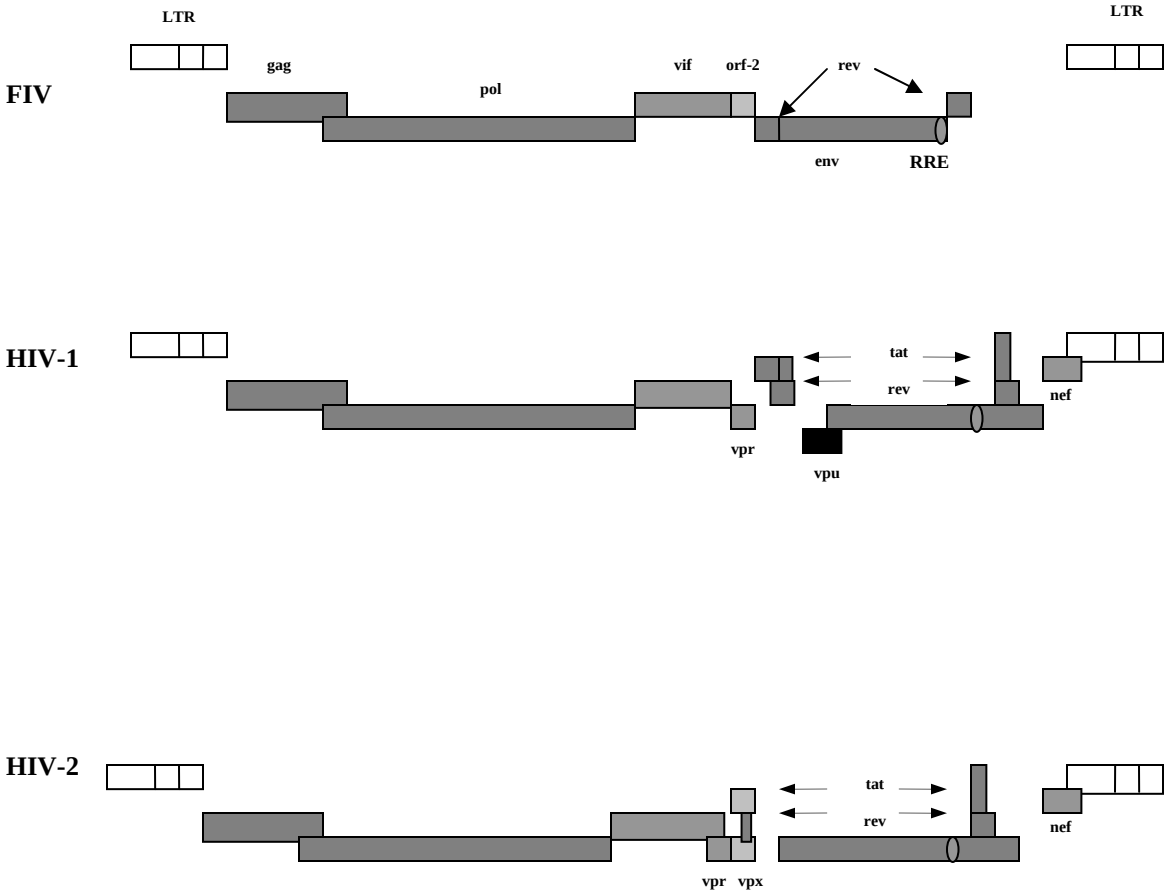
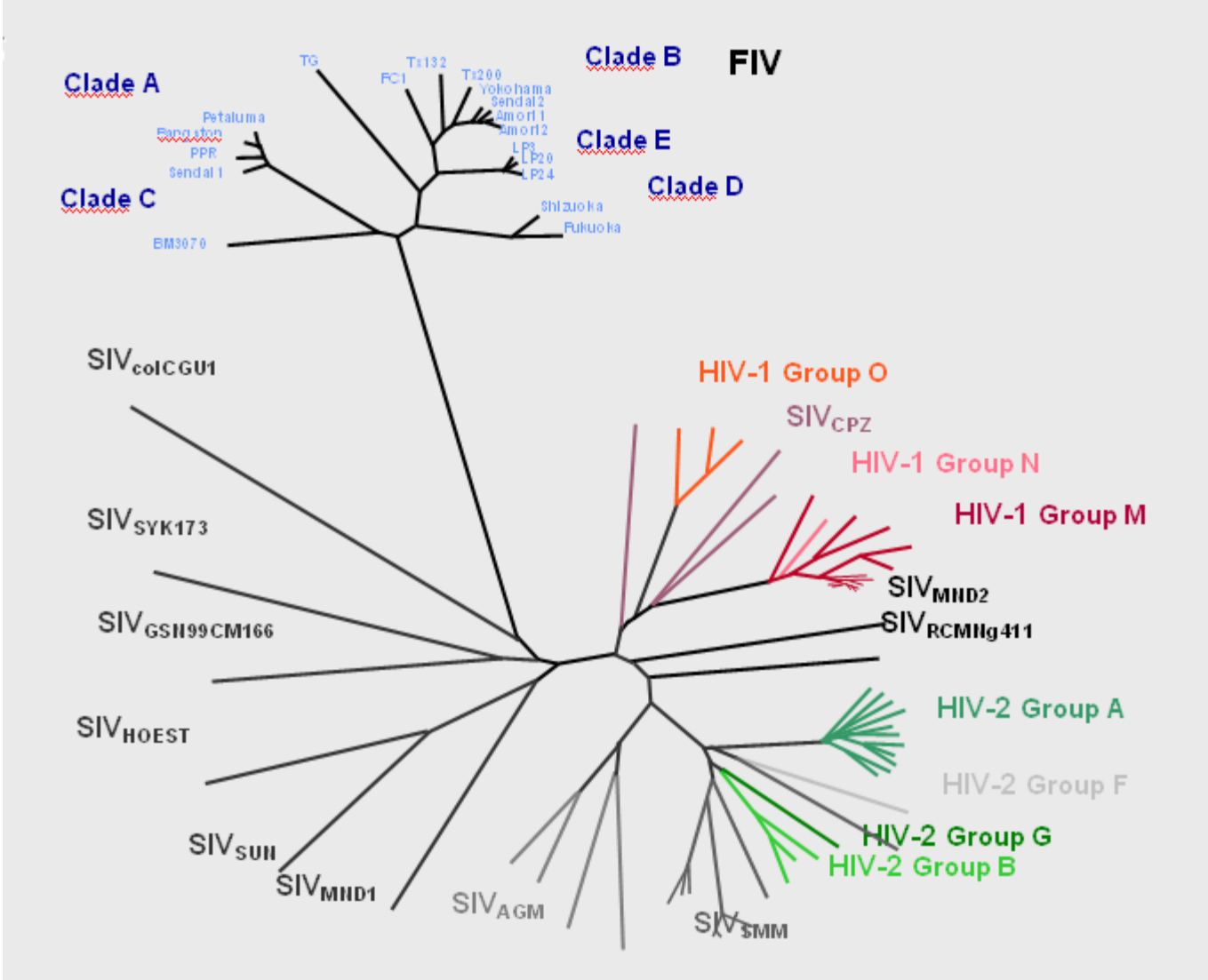


Figure 1-1. Proviral genomic organization of FIV, HIV-1, and HIV-2. FIV resembles HIV by having *LTR-gag-pol-env-LTR* genomic organization, while missing few regulatory genes (*vpu, vpx, nef*). The *orf-2* gene was initially reported to express 79 aa Tat-like protein. However, recent studies suggest that this gene may also serve as *vpr* based on its gene product promoting the transport of the DNA pre-integration complex into the cell nuclei. Diagram adapted from Sauter, S.L, et al. 2001 [7].

Figure 1-2. Comparative *gag* phylogenetic tree of FIV, HIV-1, HIV-2, and SIV. An unrooted phylogenetic tree was created using the BLOSUM matrix and neighbor-joining algorithm based on the Kimura two-parameter correction[44], [45]. Support at each internal node was assessed using 1000 bootstrap samplings and each tree was visualized using Tree View. Branch lengths, drawn to scale, are based on number of synonymous substitutions per site. The *gag* tree is based on sequences from NCBI GenBank (FIV accession numbers NC_001482, AY684181, M36968, D37820, DQ365596, AY13911, AY139112, AY139110, D37819, D37821, D37823, D37824, AF474246, AY679785, D37822, AB027302, AB027304, AB027303; SIV accession numbers AF301156, L06042, AF468659, AF075269, AF131870, M27470, AF328295, 349680, M30931, L40990, M66437, U58991, AF334679, AF077017, M80194, U72748, U79412, M32741, M33262, M19499, M83293, AJ271369, AF103818, U42720; HIV-1 accession numbers L39106, M62320, M38429, M93258, M17449, L31963, AF004394, U43096, U23487, AY679786, AF321523, AB023804, M22639, K03454, U88826, AF005496, AJ006022, L20587, AJ302647, L20571; HIV-2 accession numbers M31113, AF082339, M30502, D00835, X52223, J03654, M30895, Z48731, X05291, J04498, U22047, X62240, U27200, L07625, U75441, AF208027). HIV-1 distribution is shown in major groups (Group O for outlier group, Group M for main group; Group N for non-M group); while HIV-2 distribution is shown in subtypes (subtypes C, D, and E missing). SIV_{col}, SIV_{SYK}, SIV_{GSN}, SIV_{HOEST}, SIV_{SUN}, SIV_{MND}, SIV_{RCM}, SIV_{SM}, and SIV_{AGM} are SIV species isolated from Colobus, Sykes, greater-spot-nosed, L'Hoest, sun-tailed, mandril, red-capped mangabeys, sooty mangabeys, and African green monkeys, respectively. SIV_{mac} belongs to SIV $_{SM}$ group and are isolates from captive macaques [46]. The *gag* phylogenetic tree shows close relationship between SIV_{SM}/SIV_{mac} and HIV-2 and between SIV_{cpz} and HIV-1, but distant relationship between FIV and primate AIDS lentiviruses. This figure was adapted from Yamamoto, J.K, et al. 2005.


Clade A
Clade B
FIV
Clade C
Clade D
Clade E
TG
FC1
Tx132
Tx200
Yokohama
Sendai2
Amori1
Amori2
LP3
LP20
LP24
Petaluma
Pangaton
PPR
Sendai1
Shizuoka
Fukuoka
BM3070
SIVcolCGU1
SIVSYK173
SIVGSN99CM166
SIVHOEST
SIVSUN
SIVMND1
SIVAGM
SIVSMM
HIV-1 Group O
SIVCPZ
HIV-1 Group N
HIV-1 Group M
SIVMND2
SIVRCMNg411
HIV-2 Group A
HIV-2 Group F
HIV-2 Group G
HIV-2 Group B

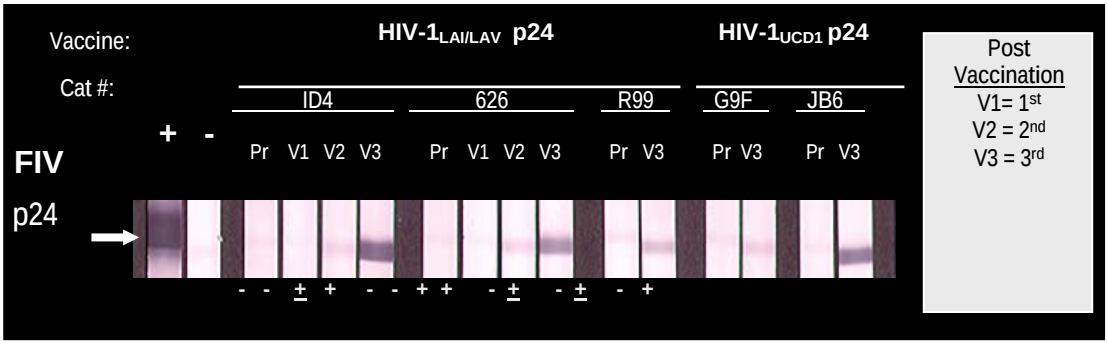


Figure 1-3. FIV p24 cross-reactivity of the sera from HIV-1 p24-vaccinated cats. FIV_{Bang} p24 strip (200 ng/strip) was reacted with individual cat serum at 1:100 dilution. Tested sera were pre- (pr) and post-3rd immunization(pre-challenge) sera of cats vaccinated with either $HIV1_{LAV}$ or $HIV1_{UCD1}$ p24 proteins. In addition, serum reactivities of post-1st and 2nd vaccinations are shown for cats #ID4 and #626. Based on immunoblot and ELISA analyses, approximately 80% of HIV-1 p24-vaccinated cats developed cross-reactive antibodies to FIV p24.

CHAPTER 2
THE ROLE OF ANTIBODY IMMUNITY IN THE PROTECTION CONFERRED BY HIV-1UCD1 P24 VACCINE

Introduction

Many of the challenges faced during the development of an FIV vaccine are common to the development of an HIV-1 vaccine [23]. Therefore, the insights derived from the development of an FIV vaccine could also lead to clues in developing an effective HIV-1 vaccine. Identifying the epitopes for vaccine protection and the immunity required for such protection are the two major aims of the proposed project on subtype-A HIV-1 p24-based FIV vaccine. This section initiates the examination of the role of vaccine-induced antibody immunity in p24 vaccine protection. Antibodies generated by vaccination were examined for patterns of FIV and HIV-1 p24 epitope recognition among protected/vaccinated cats, unprotected/vaccinated cats, and infected control cats. The vaccine immunogens consisted of subtype-A HIV-1 p24, subtype-B HIV-1 p24, FIV p24, and as a control, inactivated dual-subtype FIV whole-virus (Fel-O-Vax FIV vaccine, Fort Dodge Animal Health, Fort Dodge, Iowa). The reactivity of these sera was evaluated by western blot analysis for antigen specificity, ELISA for level of affinity to native immunogen and peptides, VNA assay for biological activity, and B-cell epitope mapping to identify vaccine epitopes of protection. Overall, the structural and functional analyses of the B-cell epitopes on the HIV-1 p24 were performed to determine the significance of HIV-1 p24 vaccine-induced antibodies in cross-protection against FIV.

In addition to the in vitro functional analysis, the vaccine-induced antibodies were tested for in vivo efficacy against FIV challenge using passive-transfer studies (Table 2-1, 2-2, and 2-3). Passive-transfer studies consisted of intravenous transfusion with vaccine-induced antibodies into naïve SPF cats one-day prior to and one day after FIV challenge (Figure 2-1). These cats were then monitored for the levels of infection and FIV-specific antibody development. Three

separate passive-transfer studies were performed. The first study was performed using pooled serum from vaccinated donor cats as the transfer material. The second study involved the use of donor antibodies, which were partially purified by ammonium sulfate precipitation method. The third study was performed using material purified by both ammonium sulfate precipitation and caprylic acid fractionation methods (Figure 2-2). The purity and activity of these antibodies were tested prior to transfusion.

In order to identify the B-cell epitopes responsible for the cross-reactive antibodies, B-cell epitope mapping was performed using sera derived from both protected and unprotected cats, which were vaccinated with HIV-1 p24. The intent of these studies was to examine the FIV p24 epitope reactivity of these different groups of animals and to determine if there was a particular profile, which was associated with infection or vaccine protection [38].

Methods

Animals

Specific pathogen free (SPF) cats were obtained from Liberty Research (Waverly, New York) or Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). All of these animals tested negative for FIV, toxoplasma, and feline leukemia virus, and they were maintained at the University of Florida experimental animal care facility under SPF conditions during vaccination or prior to FIV infection. Animals for FIV-challenge studies were transferred to BSL-2 facilities before FIV inoculation.

Serum Collection for B-cell Epitope Mapping by FIV ELISA

The animals were vaccinated subcutaneously (SC) at 3-week intervals with FIV-1_{UCD1} p24 or FIV-Petaluma (FIV_{Pet}) p24 vaccine. The vaccine consisted of the p24 protein (200-250 μg/dose) in Ribi adjuvant supplemented with 5 μg/dose of human IL-12. In addition, sera from FIV-Bangston ($FIVP_{Bang}$)-infected cats at 18 weeks post-challenge (wpc) were used as positive

control sera, while negative control sera were from uninfected SPF cats. Whole blood was centrifuged at 2000 rpm for 20 minutes, and the serum was collected and stored at -20°C until use.

Overlapping FIV P24 Peptides for ELISA

Overlapping 28-30mer peptides of FIV p24 with 11-13 aa overlaps were designed using LANL PeptGen program and synthesized by SynPrep Corp. (Dublin, California) based on the p24 aa sequence of FIV_{Bang}, with the exception of four peptides designated FB1, FB2, FC2, and FB4, which were derived from FIV_{FC1} (Figure 2-3). These were pre-screened at the company by HPLC for purity and were determined to be > 95% pure. The peptides were diluted in coating buffer (0.35 M sodium bicarbonate solution, pH 9.4). ELISA was performed by standard laboratory method [40]. ELISA plates were coated with 400 ng of the appropriate peptide in 50 µL of coating buffer per well and incubated overnight at 4°C. These wells were subsequently blocked for 1 hour with 200 µL of blocking solution (5% milk protein in PBS), washed 3X with wash buffer (150 mM NaCl and 0.05% Tween 20), and incubated with 1:200 dilution of cat serum for 1 hour at 37°C. The wells were then washed 3X with wash buffer and incubated with 100 µL of biotinylated anti-cat IgG (Vector Laboratories, Burlingame, California), diluted 1:5000 in Buffer 3 (3% BSA/PBS with 0.05% Tween-20) with 5% milk for 1 hour at 37°C. After incubation the plates were washed 3X, in wash buffer and incubated for 1 hour at 37°C with 100 µL per well strepavidin-conjugated horseradish preoxidase (Vector Laboratories). The plates were then washed 3X and 100 µL of substrate solution (0.005% tetramethylbenzidine and 0.015% H_2O_2 in 0.96% citric acid solution) was added to each well and incubated for 15 minutes. The reaction was stopped by the addition of 100 µL per well of 0.12 % hydrogen fluoride solution.

Immunoblot Analysis

The sera from vaccinated and unvaccinated cats before and after FIV challenge were tested for reactivity to immunoblot strips, each containing 200 ng of virus proteins. Immunoblot strips were incubated individually with 1:50 dilution of cat serum in Buffer 3 with 5% milk. The control sera consisted of serum from an SPF cat for negative control and serum from an FIV-infected cat for positive control. The remaining immunoblot method was performed using laboratory protocol [41].

Passive-transfer Studies with Antibodies from Vaccinated Cats

Three separate passive-transfer studies were undertaken. In Passive-Transfer Study 1, four groups of four cats were IV transfused with saline or pooled sera from either HIV-1_{UCD1} p24-vaccinated cats, Fel-O-Vax FIV-vaccinated (Fel-O-Vax-vaccinated) cats, or non-vaccinated cats. These sera were collected from the SC-vaccinated donor cats 2-3 weeks after the 3rd vaccination and before FIV challenge (vaccinated donors in Chapter 3). All pooled sera were inactivated by incubation at 56°C for 40 minutes. In order to prevent serum reaction, the pooled serum was cross-matched with each recipient cat's blood. Each cat received a cross-match-negative serum equivalent to 30% of the recipient's total blood volume. The amount of volume, including serum protein level, was too large to administer in a single transfusion. For this reason, cats received 20% volume in the first transfusion and 10% volume in the second transfusion. The first transfusion was given on day -1 with FIV challenge on day 0 and second transfer on day 1 (Figure 2-1). These cats were challenged IV with FIV_{Pet} (10 mean cat infectious dose, CID_{50}).

In Passive-Transfer Study 2, the antibodies were purified from pooled serum by ammonium sulfate precipitation, which partially purifies for IgG. SPF cats were transfused with saline or partially-purified antibodies of pooled serum from HIV-1_{UCD1} p24-vaccinated cats,

FIV_{Pet} p24-vaccinated cats, Fel-O-Vax-vaccinated cats, or non-vaccinated cats. In Passive-Transfer Study 3, the antibody purification was taken further using a two-step purification method involving caprylic acid fractionation followed by ammonium sulfate precipitation. After these procedures were completed, the antibodies were dialyzed and sterilized by 0.45 μm filtration. Tests for biological activity, purity, and toxicity were performed on the material prior to passive transfer. The vaccine specificity of the antibodies in Study 3 was identical to those of Study 2, except for the additional antibody group derived from uninfected FeT-J cell/lysate-immunized (FeT-J cell-immunized) cats. FeT-J cells were the cells used to produce the viruses for the Fel-O-Vax FIV vaccine. These cats received a combined cell and cell lysate immunization using SC ($2x10^6$ cells/dose), intradermal (250 μg/dose cell lysate), intranasal (100 μg/dose cell lysate), and transcutaneous (100 μg/dose cell lysate) routes (see Chapter 3 for additional detail). All immunizations were administered at 3-week intervals. The passive-transfer schedule and transfusion procedure for both Studies 2 and 3 were identical to the one in Study 1. Cats in Studies 2 and 3 included groups challenged IV with FIV_{FC1} (Study 2, 15 CID_{50}; Study 3, 10 CID_{50}) and another set of groups challenged IV with FIV_{Pet} (25 CID_{50}).

Antibody Preparation

Blood was collected from HIV-1_{UCD1} p24, HIV-1_{N20}, FIV p24, and FeT-J cell lysate-immunized donor cats and from SPF cats in either serum or heparinized collection tubes. The whole blood was centrifuged at 2000 rpm for 20 minutes and the serum or plasma was collected. The sera were pooled according to immunization group and purified for IgG by ammonium sulfate precipitation alone (Study 2) or in combination with caprylic acid fractionation (Study 3) (Figure 2-2). The pooled serum and purified antibody preparations were tested by immunoblot and radial immunodiffusion (RID) assay to determine the reactivity to the target immunogens

and to determine the immunoglobulin levels (IgG, IgA, IgM), respectively. Commercial RID assay for feline IgG, IgA, and IgM (Bethyl Laboratories, Montgomery, Texas) were performed according to the manufacturer's protocol.

Ammonium sulfate precipitation

The supernatant from caprylic acid purification was cooled to 4^{o}C then placed into a container and stirred slowly using a magnetic stirring bar. Ammonium sulfate was added to each sample 0.65 g/mL (Fisher Scientific Pittsburg PA) to achieve 100% saturation. Solid ammonium sulfate was slowly added to the antibodies and the mixture was stirred at 4 ^{o}C overnight. Following overnight incubation, the sample was centrifuged (20g) for 20 minutes at 4^{o}C. The solid fraction was pooled and dissolved in an equal volume of H_2O. The fractions were then dialyzed against PBS using 600-800 MW Spectra/ Por (Sprectra Laboratories, Inc., Rancho Dominguez, California) [47].

Caprylic acid purification

Serum samples were pooled for each group, transferred to a clean container, and mixed with 60 mM of sodium acetate (Sigma-Aldrich USA, St. Louis, Missouri) solution at pH 4. Each pool of antibodies was mixed with 0.75 mL caprylic acid (Sigma Aldrich USA) per 10 mL of original sample volume. This mixture was stirred for 30 minutes and then centrifuged at 5000g for 10 minutes. The supernatant was decanted and subsequently dialyzed against PBS using 600-800 MW Spectra/Por (Sprectra Laboratories. Inc.) [47]. The antibodies were then concentrated to half the previous volume using a Bio-Rad Ultra concentrator. The final product was filter purified using a 0.45 μm filter. The product was tested by western blot, ELISA, and gel analysis using Coomassie Brilliant Blue (CBB) stain and silver stain to determine purity, and by VNA assay to determine retention of biological activity [48].

Virus isolation

Whole blood was collected from the cats in passive-transfer studies at 3-4 week intervals. The peripheral blood mononuclear cells (PBMC) purified by ficoll-hypaque gradient technique [8] were used to determine the FIV infection status. These cells ($1.5x10^6$-$3x10^6$ cells) were co-cultured with indicator cells ($3x10^6$ cells) in 3 mL of culture media for approximately 3 weeks. The cells were recultured every 3 days and the spent culture fluid from each passage day was collected. The culture fluid was then tested for RT activity [7]. The indicator cells consisted of PBMC from SPF cats, which were stimulated with 5 μg/mL of concanavalin A (Con A) for 3 days and recultured every 3 days in fresh culture media for 6-12 days before use in the assay.

VNA assay

Blood from the cats in passive-transfer studies were collected pre- and post-challenge at 3-4 week intervals. The serum from blood was separated by centrifugation at 2000 rpm for 20 minutes, and the serum was collected and frozen until the day of assay. VNA assay was performed in accordance with established laboratory protocol [6]. On the day of assay, the serum was incubated at 56°C for 30 minutes to inactivate the complement. Serial dilutions of the serum in culture media were incubated with 200 $TCID_{50}$ of virus for one hour before addition of 0.25 mL mixture to $0.5x10^5$ indicator cells in 0.25 mL/well. The final serum dilutions ranged from 1:10 to 1:10,000, and the final virus titer was at 100 $TCID_{50}$. The cells were recultured every 3 days in fresh culture media for 18-21 days, while the spent culture fluid was collected and later tested for FIV levels by RT assay. The indicator cells were prepared the same way as those used in the virus isolation assay.

Results

B-cell Epitope Analysis

ELISA analysis of the reactivity of HIV-1 p24-vaccinated cat serum to the overlapping 28–30mer peptides of FIV_{Bang} p24 with 11 aa overlaps showed highest reactivity to peptide 71-100 (FB4) followed by peptide 197-223 (FB11). Less reactivity was also observed to peptide 53-81 (FB3), 161-188 (FB9), and 178-207 (FB10) (Figure 2-3). This indicates that at least 5 cross-reactive B-cell epitopes are induced by vaccination of cats with HIV-1 p24. However, these cross-reactive peptides did not correspond to the major homology regions (MHR) found among FIV, SIV, and HIV [49-52] (Figure 2-4). As expected, FIV p24-vaccinated cats had the most serum reactivity to FIV p24 peptides (11 of 12 peptides), while FIV-infected cats had a moderate-to-high frequency of serum reactivity to three FIV p24 peptides (FB1, FB4, FB11). FB4 and FB11 peptides, which reacted with the highest frequency to sera from infected cats, also reacted with highest frequency to sera from HIV-1 p24-vaccinated cats. This observation suggests that both vaccination with HIV-1 p24 and infection with FIV stimulate a similar B-cell repertoire to FIV p24 epitopes. No VNA activity was observed with antibodies generated to HIV-1 p24 and to FIV p24 (data not shown). In contrast, VNA activity was observed with sera from dual-subtype FIV-vaccinated cats (both prototype and commercial dual-subtype FIV vaccines tested, data not shown).

Passive-transfer Studies: The Analysis of Purified Antibody Preparations for Transfer

Greater than 90% of the contaminating serum proteins were removed with the use of caprylic acid/ammonium sulfate precipitation procedure (Figure 2-5). This is evident from both the CBB-gel and immunoblot analyses of the purified antibodies and the corresponding pre-purification sera from each vaccination group. Albumin was used as a measure of contamination in the purified antibody preparations. Albumin levels were determined by immunoblot analysis

using commercial polyclonal antibodies to feline albumin (Bethyl Laboratories). Purified antibody preparations contained <95% albumin when compared to the amounts in corresponding pre-purification sera (Figure 2-5). Thus, >90% of the contaminating proteins were removed according to the minor albumin contamination remaining and few weak bands of other contaminating proteins in the CBB-gels. Furthermore, the immunoblots show heavy and light chains of the cat antibodies at the predicted molecular weight sizes of 50 kD and 25 kD, respectively (Figure 2-6). The intensity of the bands for heavy and light chains suggests that high levels of antibodies were retained after purification. Analysis for IgG levels using RID assay indicates that most of the IgG in the purified antibody preparations (average recovery of 75%; recovery range of 67%-82%) were retained after the purification (Figure 2-6 and 2-7). The purified antibody preparations also contained lower amounts of IgA (average recovery of 36%; recovery range of 19-62%) and IgM (average recover of 53%; recovery range of 48-55%) than those in the pre-purification sera (Figure 2-8 and 2-9). The loss of IgA and IgM was anticipated since caprylic acid/ammonium sulfate precipitation procedure has been reported to be a purification procedure for IgG [47].

The retention of biological activity was illustrated by the reactivity of the purified antibody preparations to the vaccine immunogen used on the donors of their pre-purification pooled serum (Figure 2-10). Furthermore, no significant loss in VNA titers to FIV_{Pet} were detected after purification of pooled serum from Fel-O-Vax-vaccinated cats (Table 2-5; 1000 VNA titers of pre- and post-purification). The VNA titers to FIV_{Pet} and FIV_{FC1} were not detected in pooled serum from either HIV-1 p24- or FIV p24-vaccinated cats.

All purified antibodies were resuspended to the original volume to simulate the concentrations equivalent to those in the pre-purification pooled sera. The same dilution of

1:200 was used for the purified preparation and pre-purification serum in the immunoblot analysis. These adjustments allowed for the direct comparison between the pre- and post-purification materials. Therefore, the intensity of the bands was used as a measure for comparing specific-antibody titers in purified preparations to those in pre-purification sera. Purified antibody preparations from pooled sera of HIV-1_{UCD1}- and HIV-1_{N20} p24-vaccinated cats reacted strongly to HIV-1_{UCD1}- and HIV-1_{N20} p24 immunoblots (Figure 2-10A, 2-10B). They reacted weakly to p24 of FIV whole-virus immunoblot (Figure 2-10C). However, their weak p24 reactivity in the FIV whole-virus immunoblot was slightly stronger than their pre-purification pooled serum (Figure 2-10C). The purified antibody preparation from pooled serum of FIV p24-vaccinated cats reacted to HIV-1 p24 immunoblots at a similar weak intensity as that of corresponding pre-purification serum (Figure 2-10A, 2-10B). As expected, purified antibody preparations from sera of HIV-1 and FIV p24-vaccinated cats had no reaction to uninfected FeT-J cell-lysate immunoblot (Figure 2-10D). Whereas, purified antibody preparation and pre-purification pooled serum from Fel-O-Vax-vaccinated cats reacted weakly at similar bands (50 and 75kD on FeT-J immunoblots). These reactions to cellular components were anticipated since Fel-O-Vax FIV vaccine, which is composed of inactivated infected cells and viruses, should also induce anti-cellular antibodies.

Passive-transfer Studies with Serum and Antibodies from Vaccinated Cats

In Passive-Transfer Study 1, 3 of 4 (75%) cats transfused with HIV-1_{UCD1} p24-vaccinated cat serum became infected with FIV_{Pet} (10 CID_{50}) (Table 2-1). Whereas, Fel-O-Vax-vaccinated cat serum was able to protect 4 of 4 (100%) cats from this group. Furthermore, no protection was observed in all four control cats, which received saline or pooled serum from non-vaccinated cats.

In Passive-Transfer Study 2 with slightly higher challenge dose of 25 CID_{50}, all three cats transfused with partially-purified antibodies from pooled serum of HIV-1_{UCD1} and FIV_{Pet} p24-vaccinated cats, were not protected against FIV_{Pet} (Table 2-2). Three of 4 cats, which received similarly purified antibodies from Fel-O-Vax-vaccinated cat sera, were protected against FIV_{Pet} but not against FIV_{FC1} even at a slightly lower challenge dose (15 CID_{50}). As expected, all five recipients of saline or partially-purified antibodies from non-vaccinated cats were all infected with either FIV_{Pet} or FIV_{FC1}. These observations suggested that the antibodies induced by Fel-O-Vax FIV vaccination can confer protection against homologous FIV challenge but not against heterologous-subtype FIV_{FC1}. Furthermore, antibodies induced by HIV-1 or FIV p24 vaccination conferred no protection against FIV_{Pet}.

In Passive-Transfer Study 3, 1 of 3 cats transfused with highly-purified antibodies from HIV-1_{UCD1} p24-vaccinated cat sera were protected against FIV_{FC1} (10 CID_{50}), while no protection (0 of 4) was observed in recipients of purified antibodies from HIV-1_{N20} p24-vaccinated cat sera (Table 2-3). Moreover, all four cats transfused with purified antibodies from either FIV p24-vaccinated (n=2) or Fel-O-Vax-vaccinated (n=2) cat sera were not protected against FIV_{FC1}. Like the results from Study 2, all four cats transfused with purified antibodies from Fel-O-Vax-vaccinated cat sera were protected against homologous FIV_{Pet}. VNA analysis of antibodies removed from the transfused cats 6 days post transfusion showed the retention of some VNA activity at an average dilution of 1:800 (800 VNA titer). All recipients of saline or purified antibodies from FeT-J cell-immunized or non-vaccinated cat sera were infected with either FIV_{Pet} or FIV_{FC1}. Hence, the antibodies to HIV-1 p24 and FIV p24 do not mediate the protection observed in HIV-1 p24-vaccinated cats and FIV p24-vaccinated cats.

Discussion

As summarized in Table 2-4, current studies demonstrated that only 1 of 8 cats transfused with serum or antibodies to HIV-1_{UCD1} p24, 0 of 3 cats transfused with antibodies to FIV p24, and 11 of 12 cats transfused with antibodies to Fel-O-Vax antigen were protected against FIV_{Pet} challenge. In contrast, 1 of 3 cats transfused with antibodies to HIV1_{UCD1} p24 and none of the cats transfused with antibodies to HIV-1_{N20} p24, FIV p24, or Fel-O-Vax immunogen were protected against FIV_{FC1} challenge. These challenge doses resulted in infection of all control cats, which received saline, antibodies to FeT-J cells, or antibodies from SPF cats. Hence, no significant levels of passive-transfer protection were observed in cats transfused with antibodies to HIV-1 p24 or FIV p24. However, passive-transfer protection was conferred with antibodies from Fel-O-Vax-vaccinated cats against homologous FIV_{Pet} challenge but not against FIV_{FC1} challenge. These results taken together suggested that cross-protection, previously observed in HIV-1 p24-vaccinated cats against both FIV_{Pet} and FIV_{FC1} and in Fel-O-vax-vaccinated cats against FIV_{FC1} was not mediated by vaccine-induced antibodies.

Antibodies to HIV-1 p24 and FIV 24 conferred no passive-transfer protection. This finding was anticipated since VNAs to p24 have yet to be reported. Only antibodies to SU and TM, which are exposed on the viral surface, have been reported [53]. The core p24 is masked by the viral lipid envelope and blocked from interaction with the antibodies to p24. This also explained why only antibodies induced by Fel-O-Vax FIV vaccination, which had high levels of antibodies to FIV SU and TM, conferred passive-transfer protection against FIV_{Pet}. High VNA titers to FIV_{Pet}, but not to FIV_{FC1}, were observed only in the serum or purified antibodies from Fel-O-Vax-vaccinated cats (Table 2-5). This observation suggested a correlation of passive-transfer protection with high vaccine-induced VNAs to the challenge strain. Previous studies using sera from inactivated FIV_{Pet} (single-strain)-vaccinated cats and inactivated sera from

FIV_{Pet}-infected cats demonstrated positive correlation between passive-transfer protection and transfusion with sera containing high VNA titers to the challenge virus [54]. Similar observations were made in passive-transfer studies of macaques with inactivated infect sera against the homologous SIV strain [55] or with human monoclonal VNA to HIV-1 Env against a challenge with SHIV, which had homologous HIV-1 Env [56]. Thus, vaccine-induced antibodies may play a role in the protection of Fel-O-Vax-vaccinated cats against vaccine-induced VNA-sensitive, challenge viruses.

The pattern of passive-protection observed in Studies 1 and 2 with pooled serum and partially-purified antibodies was similar to the protection pattern observed in Study 3 with highly-purified antibodies. The highly-purified antibody preparations were produced by a combination of caprylic acid fractionation and ammonium sulfate precipitation. Such purification procedure produced antibody preparations with high levels of IgG antibodies (IgG levels by RID assay), biological activities (VNA activity and reactivity to HIV/FIV p24 on immunoblot), and purity (>90% purity and <50 EU endotoxin activity). The removal of serum albumin and other serum proteins were greatly reduced and had no affect on the results of the passive-transfer studies. This observation indicated that the antibodies, and not other serum proteins, were responsible for the passive-transfer protection observed against FIV_{Pet} challenge in the recipients of Fel-O-Vax-vaccinated cat antibodies. These studies do not however determine which anti-FIV immunoglobulin isotypes are more important for passive-transfer protection as well as for protection observed after active vaccination. The high retention of IgG antibodies and VNA titers in highly-purified preparations suggest that FIV-specific IgG antibodies play a major role in both passive-transfer protection and vaccine protection against homologous FIV_{Pet} challenge. This protection against IV homologous challenge may also extend towards

mucosal/vaginal challenge, since a recent vaccine study using a combination of Fel-O-Vax and prototype vaccines demonstrated major protection against vaginal FIV_{Pet} challenge [57]. High levels of IgG, but very low levels of IgA, were found in the vaginal vault of the vaccinated cats. Furthermore, high levels of FIV-specific IgG antibodies, but only low levels of FIV-specific IgA antibodies, were detected in the vaginal vault. Thus, the role of vaccine-induced IgG antibodies appeared to be more important than IgA antibodies at least in the vaginal homologous challenge.

The lack of passive-transfer protection with antibodies to HIV-1 p24 and FIV p24 suggested that vaccine protection observed in previous studies with HIV-1 p24-vaccinated cats and FIV p24-vaccinated cats [38] may be due to vaccine-induced cellular immunity. In previous studies FIV 24-specific IFNγ responses have been detected in PBMC from HIV-1 p24-vaccinated cats [38]. Similarly, cellular immunity may have mediated the protection observed in Fel-O-Vax-vaccinated cats against VNA-resistant FIV_{FC1}. Strong FIV-specific cellular immunity has been reported to be present in cats vaccinated with prototype dual-subtype FIV vaccine (prototype to Fel-O-Vax vaccine) [48]. These observations further support the view that HIV-1 p24 vaccination induces cross-protective cellular immunity against FIV challenge. The studies in Chapter 3 were performed to test this concept.

Table 2-1. Passive-Transfer Study 1 with sera from vaccinated and non-vaccinated cats

Group	Cat #	Passive-Transfer Serum	FIV Challenge (CID_{50}) [a]	Weeks Post-Challenge (FIV Ab/VI) [b] 3	6	9	12	15	17	20	Protection Rate (%)
A	RT2	$HIV1_{UCD1}$ p24-vaccinated cat serum	Pet (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1/4 (25%)
	RW1	$HIV1_{UCD1}$ p24-vaccinated cat serum	Pet (10)	-/+	+/+	+/+	+/+	+/+	+/+	Eu	
	RX3	$HIV1_{UCD1}$ p24-vaccinated cat serum	Pet (10)	-/-	+/+	+/+	+/+	+/+	+/+	Eu	
	RV1	$HIV1_{UCD1}$ p24-vaccinated cat serum	Pet (10)	-/-	-/+	+/+	+/+	+/+	+/+	Eu	
B	RT5	Fel-O-Vax FIV-vaccinated cat serum	Pet (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	4/4 (100%)
	RW2	Fel-O-Vax FIV-vaccinated cat serum	Pet (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	EF3	Fel-O-Vax FIV-vaccinated cat serum	Pet (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	EH2	Fel-O-Vax FIV-vaccinated cat serum	Pet (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
C	RT4	Non-vaccinated cat serum	Pet (10)	+/+	+/+	+/+	+/+	+/+	+/+	+/+	0/4 (0%)
	RX1	Non-vaccinated cat serum	Pet (10)	-/-	-/-	-/+	-/+	-/+	+/+	+/+	
	RW4	Saline	Pet (10)	-/-	+/-	+/+	+/+	+/+	+/+	+/+	
	DQ1	Saline	Pet (10)	-/-	-/-	+/+	+/+	+/+	+/+	Eu	

[a] FIV challenge was subtype-A FIV_{Pet} (Pet) at 10 CID_{50}.

[b] FIV infection was based on the development of FIV antibodies (FIV Ab) determined by FIV-immunoblot analysis and the presence of viruses determined by virus isolation (VI) using RTand PCR analyses. Results for the samples collected at 3, 6, 9, 12, 15, 17, and 20 wpc are shown either as positive (+) or negative (-) for FIV antibodies or viruses. Abbreviation is euthanized (Eu).

Table 2-2. Passive-Transfer Study 2 with partially-purified antibodies from vaccinated and non-vaccinated cat sera

Group	Cat #	Passive-Transfer Antibody	FIV Challenge (CID_{50})[a]	Weeks Post-Challenge (FIV Ab/VI)[b] 3	6	9	12	15	17	20	Protection Rate (%)
A	QC3	$HIV1_{UCD1}$ p24-vaccinated cat Ab	Pet (25)	-/+	+/+	+/+	+/+	+/+	+/+	+/+	0/4 (0%)
	QD3	$HIV1_{UCD1}$ p24-vaccinated cat Ab	Pet (25)	-/+	+/+	+/+	+/+	+/+	+/+	+/+	
	QE4	$HIV1_{UCD1}$ p24-vaccinated cat Ab	Pet (25)	-/+	+/+	+/+	+/+	+/+	+/+	+/+	
	BDP	$HIV1_{UCD1}$ p24-vaccinated cat Ab	Pet (25)	-/+	+/+	+/+	+/+	+/+	+/+	+/+	
B	QC1	FIV_{Pet} p24-vaccinated cat Ab	Pet (25)	-/+	-/+	-/+	-/+	+/+	+/+	+/+	0/3 (0%)
	QD4	FIV_{Pet} p24-vaccinated cat Ab	Pet (25)	-/+	-/+	-/+	-/+	-/+	+/+	+/+	
	QE7	FIV_{Pet} p24-vaccinated cat Ab	Pet (25)	-/+	-/+	-/+	-/+	+/+	+/+	+/+	
C	QC2	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	3/4 (75%)
	QD2	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/+	-/+	-/+	-/+	-/+	+/+	+/+	
	QE1	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	BDQ	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
D	QD5	Non-vaccinated cat Ab	Pet (25)	-/+	+/+	+/+	+/+	+/+	+/+	+/+	0/3 (0%)
	BDS	Non-vaccinated cat Ab	Pet (25)	-/-	+/-	+/+	+/+	+/+	+/+	+/+	
	QE2	Saline	Pet (25)	-/-	-/-	+/+	+/+	+/+	+/+	+/+	
E	QE2	Fel-O-Vax FIV-vaccinated cat Ab	FC1 (15)	-/-	-/-	-/+	-/+	-/-	+/-	+/+	0/3 (0%)
	PQ6	Fel-O-Vax FIV-vaccinated cat Ab	FC1 (15)	-/-	+/+	+/+	+/+	+/+	+/+	+/+	
	75B	Fel-O-Vax FIV-vaccinated cat Ab	FC1 (15)	-/-	+/+	+/+	+/+	+/+	+/+	+/+	
F	QE6	Non-vaccinated cat Ab	FC1 (15)	-/-	-/-	+/+	+/+	+/+	+/+	+/+	0/2 (0%)
	75C	Saline	FC1 (15)	+/+	+/+	+/+	+/+	+/+	+/+	+/+	

[a] FIV challenge was subtype-A FIV_{Pet} (Pet) at 25 CID_{50} and subtype-B FIV_{FC1} (FC1) at 15 CID_{50}.

[b] FIV infection was based on the development of FIV antibodies (FIV Ab) determined by FIV-immunoblot analysis and the presence of viruses determined by virus isolation (VI) using RTand PCR analyses. Results for the samples collected at 3, 6, 9, 12, 15, 17, and 20 wpc are shown either as positive (+) or negative (-) for FIV antibodies or viruses.

Table 2-3. Passive-Transfer Study 3 with purified antibodies from vaccinated and non-vaccinated cat sera.

Group	Cat #	Passive-Transfer Antibody	FIV Challenge (CID_{50})[a]	Weeks Post-Challenge (FIV Ab/VI)[b] 3	6	9	12	15	17	20	Protection Rate (%)
A	LF3	$HIV1_{UCD1}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1/3 (33%)
	KA4	$HIV1_{UCD1}$ p24-vaccinated cat Ab	FC1 (10)	-/-	+/-	+/-	+/+	+/+	+/+	+/+	
	KE2	$HIV1_{UCD1}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	-/-	+/+	+/+	
B	LK2	$HIV1_{N20}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	-/+	+/+	+/+	0/4 (0%)
	KA6	$HIV1_{N20}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	+/-	+/+	+/+	+/+	+/+	
	KE3	$HIV1_{N20}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	+/-	+/+	+/+	
	KB2	$HIV1_{N20}$ p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	-/-	+/+	+/+	
C	KE4	FIV_{Pet} p24-vaccinated cat Ab	FC1 (10)	-/-	+/-	+/+	+/+	+/+	+/+	+/+	0/2 (0%)
	LK4	FIV_{Pet} p24-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/-	-/-	+/+	+/+	
D	KE5	Fel-O-Vax FIV-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	-/+	-/+	+/+	+/+	0/2 (0%)
	KA2	Fel-O-Vax FIV-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/+	-/+	+/+	+/+	+/+	
E	KE6	FeT-J cell-immunized cat Ab	FC1 (10)	-/-	-/-	-/-	+/+	+/+	+/+	+/+	0/3 (0%)
	LF2	Non-vaccinated cat Ab	FC1 (10)	-/-	-/-	-/-	+/+	+/+	+/+	+/+	
	LD5	Saline	FC1 (10)	-/-	-/-	+/+	+/+	+/+	+/+	+/+	
F	JX2	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	4/4 (100%)
	LE5	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	KB4	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	JY1	Fel-O-Vax FIV-vaccinated cat Ab	Pet (25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
G	LE6	FeT-J cell-immunized cat Ab	Pet (25)	-/-	+/-	+/+	+/+	+/+	+/+	+/+	0/4 (0%)
	KB3	FeT-J cell-immunized cat Ab	Pet (25)	-/-	+/+	+/+	+/+	+/+	+/+	+/+	
	JX1	Non-vaccinated cat Ab	Pet (25)	-/-	+/+	+/+	+/+	+/+	+/+	+/+	
	KB1	Saline	Pet (25)	-/-	+/+	+/+	+/+	+/+	+/+	+/+	

[a] FIV challenge was subtype-A FIV_{Pet} (Pet) at 25 CID_{50} and subtype-B FIV_{FC1} (FC1) at 10 CID_{50}.

[b] FIV infection was based on the development of FIV antibodies (FIV Ab) determined by FIV-immunoblot analysis and the presence of viruses determined by virus isolation (VI) using RT and PCR analyses. Results for the samples collected at 3, 6, 9, 12, 15, 17, and 20 wpc are shown either as positive (+) or negative (-) for FIV antibodies or viruses.

Table 2-4. Summary of the results from Passive-Transfer Studies 1-3.

Donor Antibodies	Challenge Virus	
	$FIV_{Petaluma}$	FIV_{FC1}
$HIV\text{-}1_{UCD\text{-}1}$ p24	1/8	1/3
$HIV\text{-}1_{N20}$ p24	ND[1]	0/4
FIV_{Pet} p24	0/3	0/2
Fel-O-Vax FIV	11/12	0/5
FeT-J cells	0/2	0/1
Non-vaccinated [b]	0/5	0/2
Saline[c]	0/4	0/2

[a] Donor antibodies represent a combination of partially purified, highly purified, and unpurified serum antibodies.

[b] Donor antibodies were from non-vaccinated cats.

[c] Recipient cats were infused with saline and did not receive donor antibodies.

Table 2-5. VNA titers of highly purified antibodies and corresponding pooled serum

Pooled serum for passive transfer	Purification [a]	VNA to FIV_{Pet}	VNA to FIV_{FC1}
$HIV1_{UCD1}$ p24-vaccinated cat serum	Pre	<10	<10
	Post	<10	<10
$HIV1_{N20}$ p24-vaccinated cat serum	Pre	<10	<10
	Post	<10	<10
FIV p24-vaccinated cat serum	Pre	<10	<10
	Post	<10	<10
Fel-O-Vax FIV-vaccinated cat serum	Pre	1000	10
	Post	1000	U[b]

[a] Pre-purification (Pre) samples represent pooled serum from SPF cats immunized with the corresponding vaccine. Post-purification (Post) preparations represent highly purified antibodies, which were purified by caprylic acid fractionation followed by ammonium sulfate precipitation.

[b] Retesting or under investigation (U)

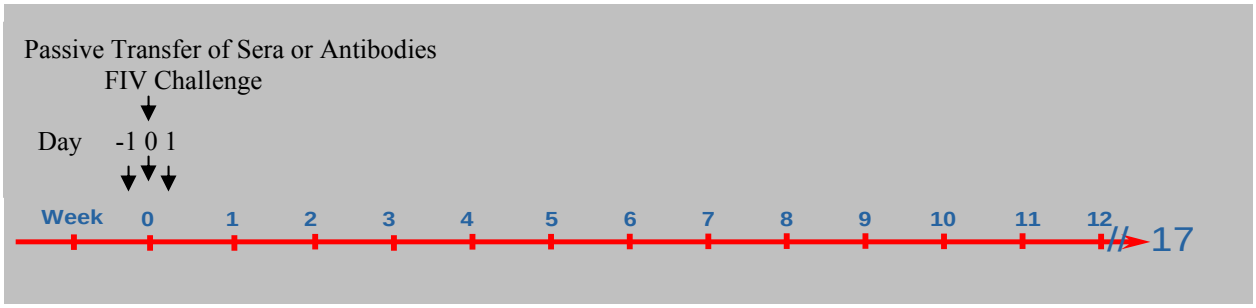


Figure 2-1. Schedule of vaccination and challenge for passive-transfer studies. Naïve SPF cats were transfused with antiserum or purified antibodies equivalent to 30% of recipient's total blood volume (TBV). First transfusion was given on day -1 (equivalent to 20% TBV) with FIV challenge on day 0 and a second transfer on day 1(equivalent to 10% TBV).

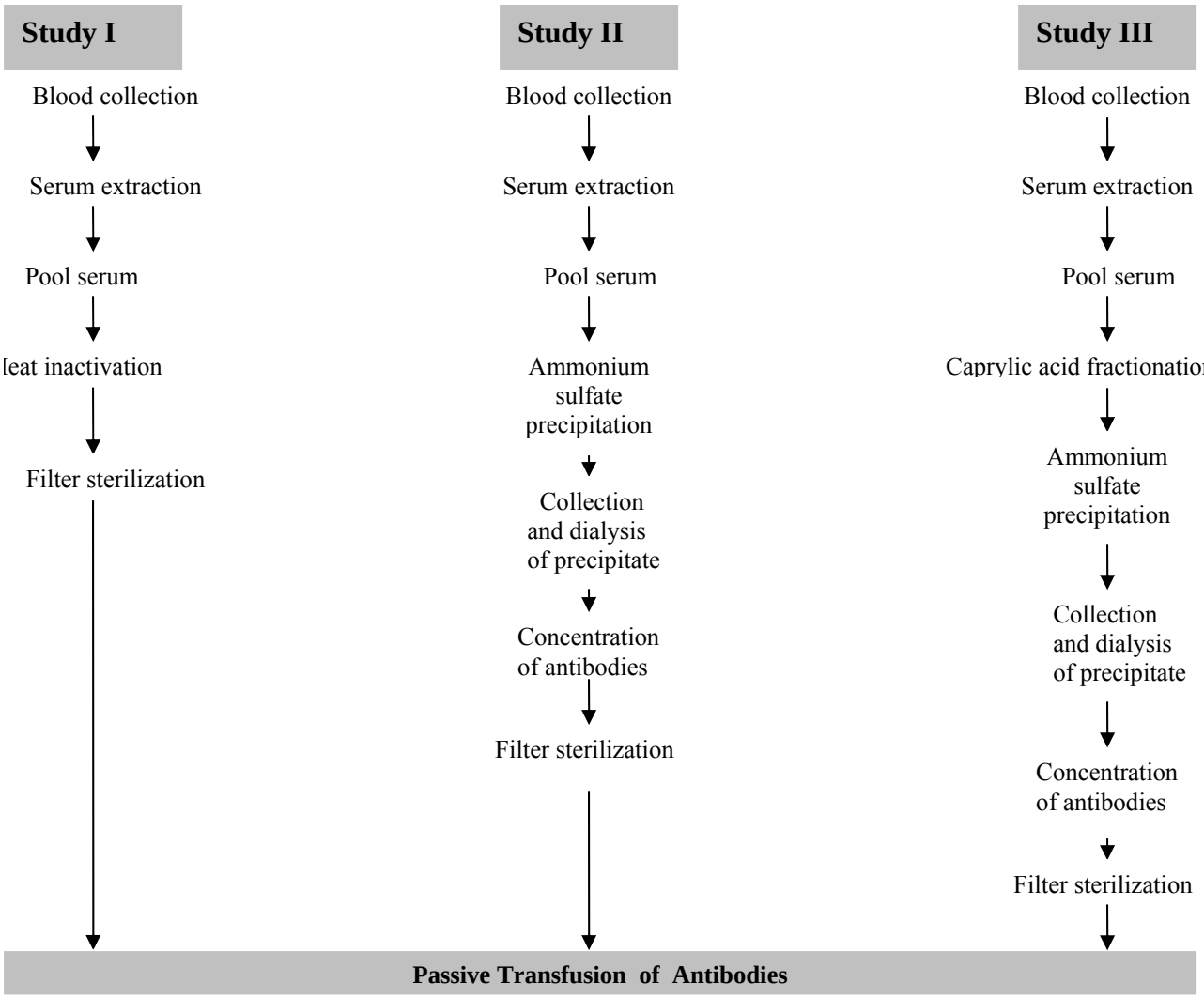


Figure 2-2. Schematic of antibody preparation for passive-transfer studies. The figure illustrates the procedure of three separate passive immunity-transfer studies. In the first study four groups of two cats each were transfused with IWV (inactivated whole virus) antiserum, Fel-O-Vax antiserum, SPF serum and saline solution. All antibodies were collected prior to the donor cats being exposed to FIV. The donor and recipients were cross-matched before use of the serum. The serum was inactivated by incubation at 56°C for 40 minutes. In the second study, the antibodies were purified by ammonium sulfate precipitation in order to isolate primarily IgG. Whilst in the third study, this purification was taken further using a two-step purification method involving caprylic acid separation followed by ammonium sulfate precipitation. After these procedures were completed the antibodies were dialyzed and filtered using a 0.45µm filter. Tests for biological activity, purity, and toxicity were performed on the material prior to passive transfer.

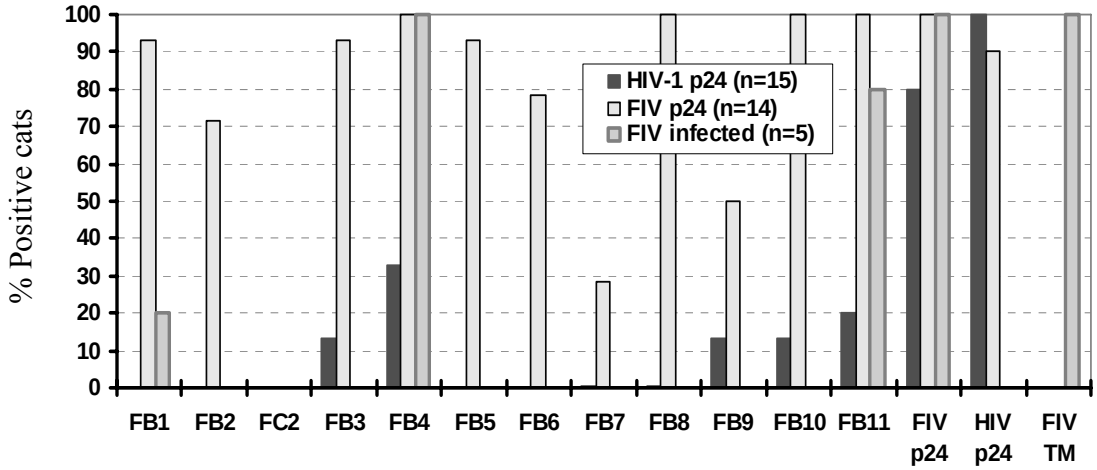


Figure 2-3. B-cell epitope analyses of sera and PBMC from HIV-1 p24-vaccinated cats. Sera from HIV-1 p24-vaccinated cats (n = 15) and FIV p24-vaccinated cats (n= 14) at 3 weeks after the 3rd vaccination before challenge and from FIV-infected control cats (n = 5) at 18 wpc were tested for reactivity to 12 overlapping B-cell peptides by ELISA. These overlapping peptides derived from FIV p24 sequence are shown with their peptide designation. The reactivity of the sera is shown as percent positive (e.g., number of positive sera among total number of sera tested). The B-cell peptide codes (without aa sequence designation) are shown below the corresponding bars the aa sequence designations are shown in Figure 2-4. As positive ELISA substrate controls, serum reactivity to FIV_{Bang} p24 protein, $HIV\text{-}1_{UCD1}$ p24 protein, and FIV transmembrane peptide TM (695–705) were determined.

Figure 2-4. Amino acid sequence alignment of HIV-1 and FIV strains, and B-cell peptide sequence alignment**.** Sequence homology analysis between HIV-1_{UCD1} and FIV_{Bang} is shown with (·) for amino acid homology and (:) for amino acid identity according to the GeneStream Align program (A). Only aa residues that are different from HIV-1_{UCD1} and FIV_{Bang} are shown for HIV-$1_{LAI/LAV}$ (above HIV-1_{UCD1} sequence) and FIV_{FC1} (below FIV_{Bang} sequence), respectively. The designations of FIV B-cell peptides and HIV-1/FIV T-cell peptides (peptide code with aa position) are shown on the top left (B). Each bar below the aa alignment represents an overlapping 28–30-mer B-cell peptide, which was used in our ELISA analysis for cross-reactive B-cell epitope mapping. The B-cell peptide overlaps by 11–13 aa, except for the four peptides (FB1, FB2, FC2, FB4) on the amino-terminus. The 26-mer peptide FC2/34–59 is based on FIV_{FC1} sequence and differs from FIV_{Bang} sequence by having Ser48 and Ser57 instead of Pro48 and Ala57. HIV-1 and FIV peptide sequences used for T-cell epitopes are shown with red aa. All 18 T-cell peptides (9–11-mers) are boxed with dotted line. T-cell peptide code is shown immediately above (HIV-1 p24 peptide) or below (FIV p24 peptide) the dotted box. Overlapping red bars at the carboxyl-terminus represent two overlapping T-cell epitopes and their corresponding sequences. HIV-1 T-cell peptides are identical to the HIV-1_{UCD1} peptide sequences. FIV T-cell peptides, except for the four peptide sequences below the alignment (F4/73–83, F4.5/122–131, F5/142–150, F7/183–191), are identical to the FIV_{Bang} peptide sequences. FIV peptides F4/73–83 and F7/183–191 are identical to the corresponding FIV_{FC1} peptide sequences and remaining two FIV peptides, F4.5/122–131 and F5/142–150, are identical to the FIV_{Shi} peptide sequences. The published major homology region (MHR) is boxed with a solid line. This figure is a modification of our figure in Coleman, J., et al. 2005 [38].

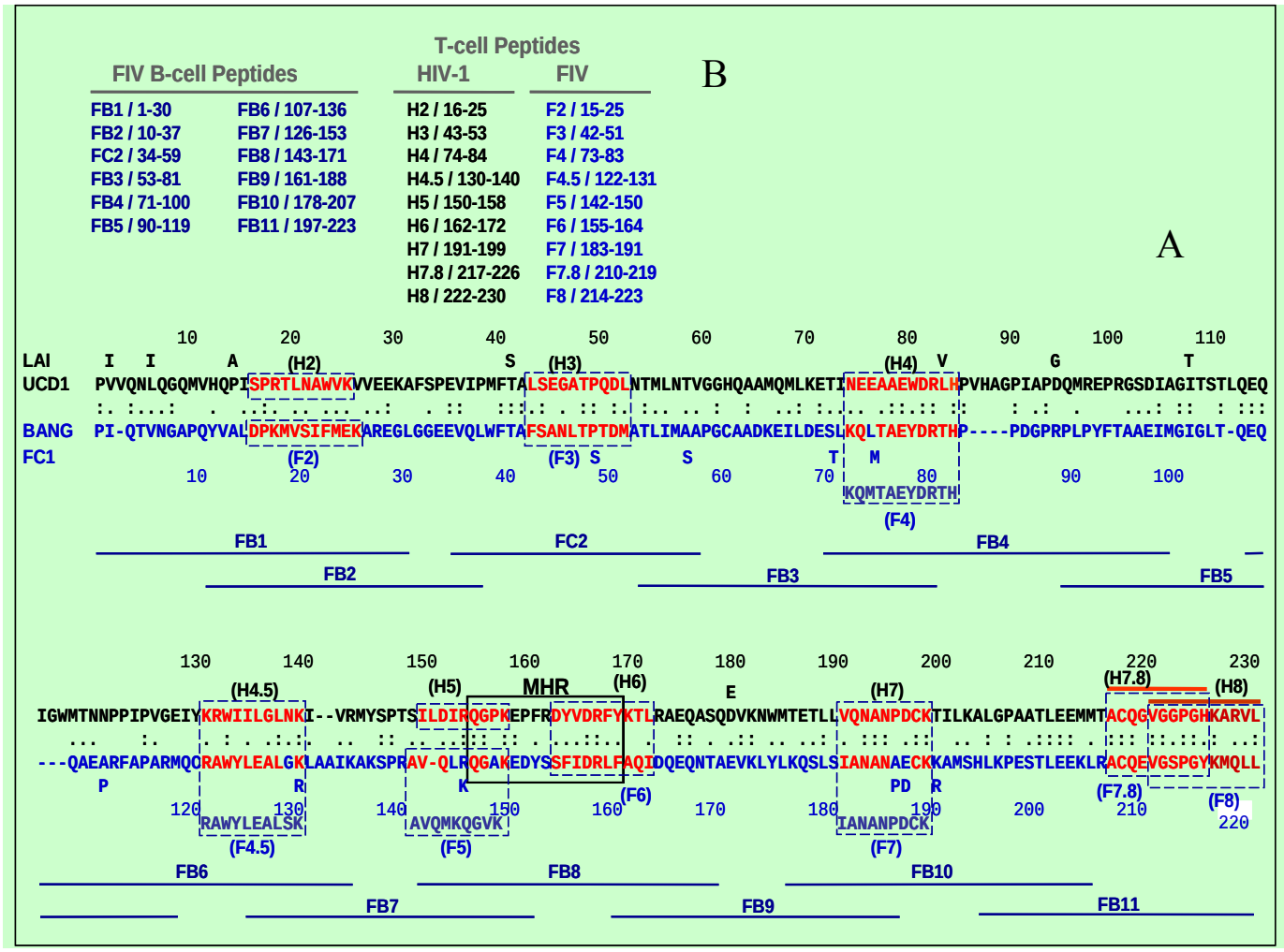
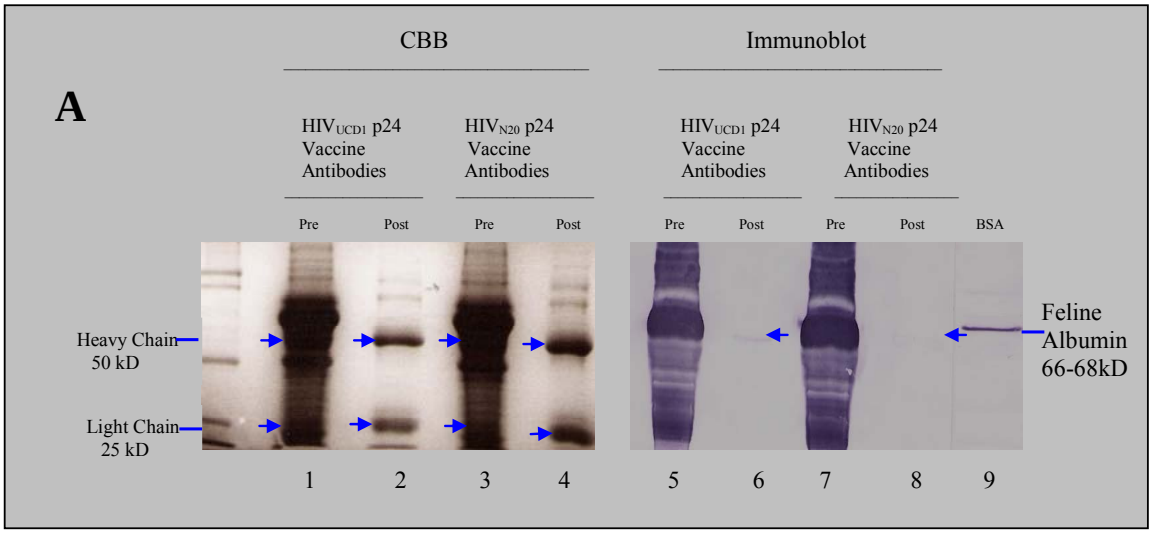
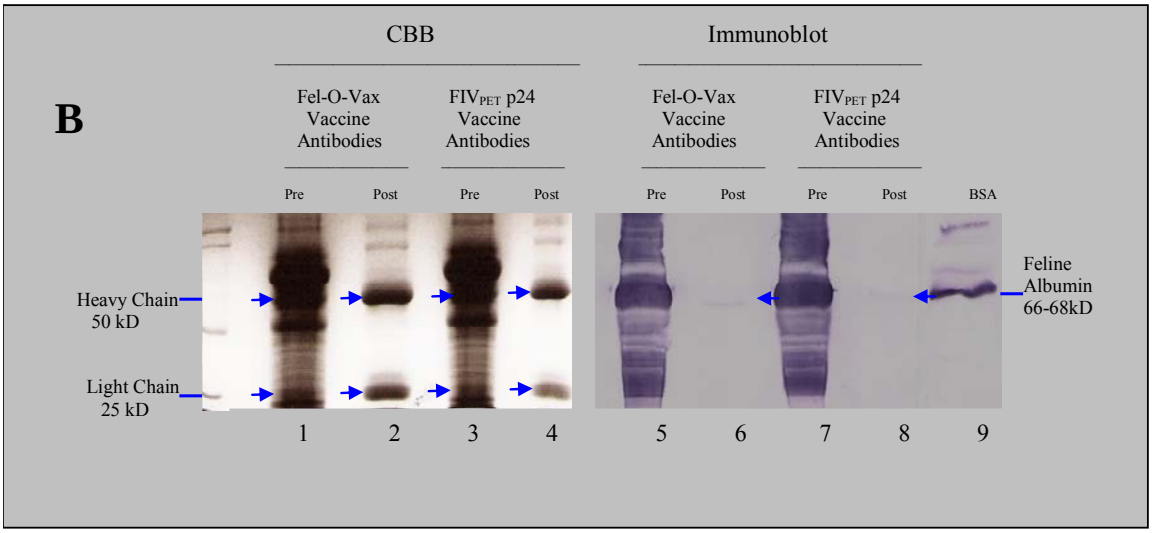
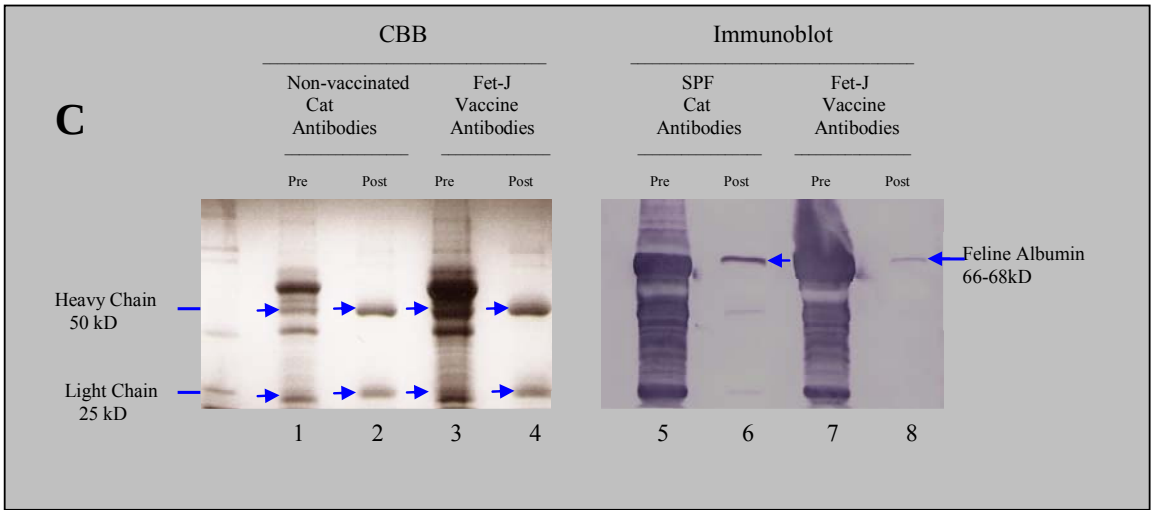

T-cell Peptides
FIV B-cell Peptides
HIV-1
FIV
B
FB1 / 1-30
FB2 / 10-37
FC2 / 34-59
FB3 / 53-81
FB4 / 71-100
FB5 / 90-119
FB6 / 107-136
FB7 / 126-153
FB8 / 143-171
FB9 / 161-188
FB10 / 178-207
FB11 / 197-223
H2 / 16-25
H3 / 43-53
H4 / 74-84
H4.5 / 130-140
H5 / 150-158
H6 / 162-172
H7 / 191-199
H7.8 / 217-226
H8 / 222-230
F2 / 15-25
F3 / 42-51
F4 / 73-83
F4.5 / 122-131
F5 / 142-150
F6 / 155-164
F7 / 183-191
F7.8 / 210-219
F8 / 214-223
A
LAI
UCD1
BANG
FC1
(H2)
(H3)
(H4)
(F2)
(F3)
(F4)
KQMTAEYDRTH
FB1
FC2
FB4
FB2
FB3
FB5
(H4.5)
(H5)
MHR
(H6)
(H7)
(H7.8)
(H8)
(F6)
(F7.8)
(F8)
RAWYLEALSK
AVQMKQGVK
IANANPDCK
(F4.5)
(F5)
(F7)
FB6
FB8
FB10
FB7
FB9
FB11

Figure 2-5. CBB-stained gel and immunoblot of purified antibody preparations for Passive-Transfer Study 3. Purified antibodies from HIV-1_{UCD1} p24 (panel A, lanes 1,2,5,6), HIV-1_{N20} (panel A, lanes 3,4,8,9) Fel-O-Vax FIV (panel B, lanes 1,2,5,6), FIV p24 (panel B, lanes 3,4,8,9), and FeT-J cell (panel C, lanes 3,4,7,8) vaccinated cat sera and non-vaccinated cat sera (panel C, lanes 1,2,5,6) are shown. Pre-purification pooled serum (Pre, 1,3,5,7) at 4 mg/lane and post-purification antibody preparation (Post, lanes 2,4,6,8) at 2 mg/lane were used in both CBB gels and immunoblots. All purified preparations in the CBB gel had strong heavy and light chain bands and weak bands at 150 kD and 100 kD, which was most likely due to unreduced and partially-reduced antibodies. The bands were also present in the immunoblot reacted to anti-feline IgG heavy and light chain in Figure 2-6. The antibodies in the immunoblots were detected using goat anti-cat albumin antibodies. Bovine serum albumin (BSA) (500 μg/lane) was used as positive control for the immunoblots (panels A and B, lane 9). A weak band at 70 kD on lane 9 were those to BSA. Feline albumin contamination was highest in the non-vaccinated cat antibody preparation. Arrows are placed next to lanes 6 and 8 of immunoblots indicate the minimal amount of feline albumin in the purified product.


A
CBB
Immunoblot
HIVUCD1 p24 Vaccine Antibodies
HIVN20 p24 Vaccine Antibodies
HIVUCD1 p24 Vaccine Antibodies
HIVN20 p24 Vaccine Antibodies
Pre
Post
Pre
Post
Pre
Post
Pre
Post
BSA
Heavy Chain 50 kD
Light Chain 25 kD
Feline Albumin 66-68kD
1
2
3
4
5
6
7
8
9


B
CBB
Immunoblot
Fel-O-Vax Vaccine Antibodies
FIVPET p24 Vaccine Antibodies
Fel-O-Vax Vaccine Antibodies
FIVPET p24 Vaccine Antibodies
Pre
Post
Pre
Post
Pre
Post
Pre
Post
BSA
Heavy Chain 50 kD
Light Chain 25 kD
Feline Albumin 66-68kD
1
2
3
4
5
6
7
8
9


C
CBB
Immunoblot
Non-vaccinated Cat Antibodies
Fet-J Vaccine Antibodies
SPF Cat Antibodies
Fet-J Vaccine Antibodies
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Heavy Chain 50 kD
Light Chain 25 kD
Feline Albumin 66-68kD
1
2
3
4
5
6
7
8

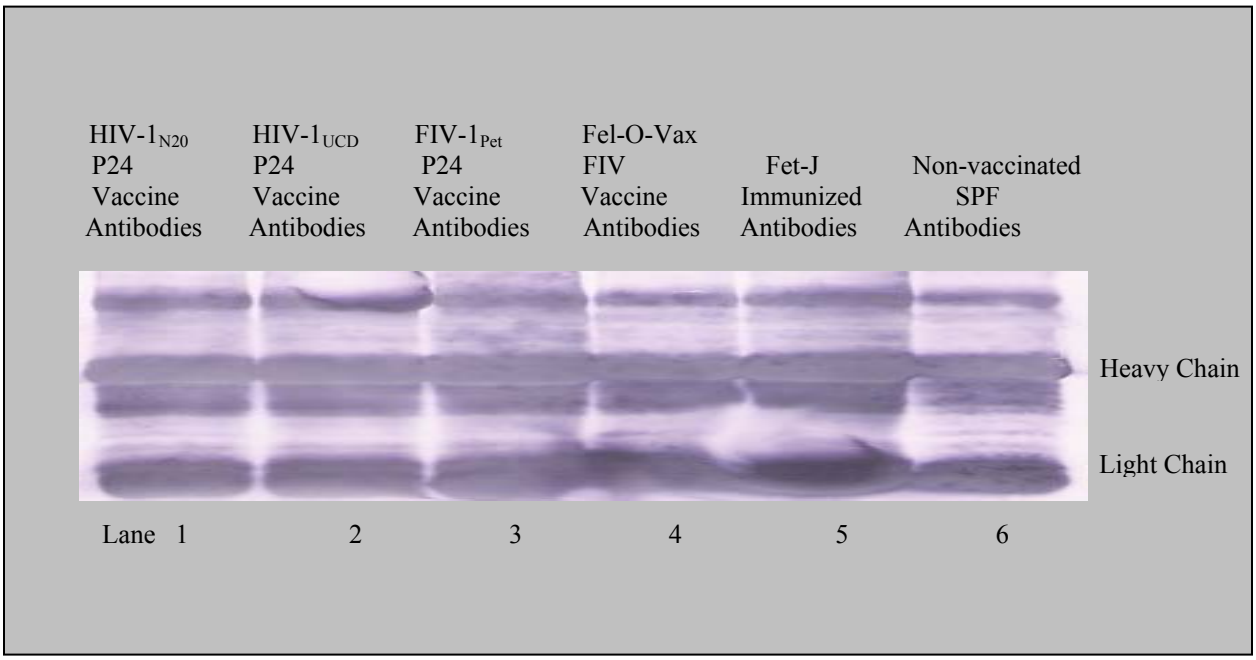


Figure 2-6. Immunoblot of purified antibody preparations used in the six groups from Passive-Transfer Study 3. Each lane contained 2 mg of purified preparation. The antibodies were detected using goat anti-cat IgG heavy and light chain antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland). Purified antibodies from HIV-1_{N20} p24 (lane 1), HIV-1_{UCD1} p24 (lane 2), FIV p24 (lane 3), Fel-O-Vax FIV (lane 4), and FeT-J cell (lane 5) vaccinated cat sera and from non-vaccinated SPF cat sera (lane 6) are shown. The molecular weight marker indicated that the bands corresponded to the predicted sizes for feline heavy and light chains. All purified antibody preparations had weaker band at 100 kD, which may be the partially-reduced immunoglobulin chain (2 heavy chains with disulphide bonds intact). The anti-cat IgG heavy/light chain antibodies showed no cross-reactivity to sheep serum, mouse serum, or BSA (data not shown).

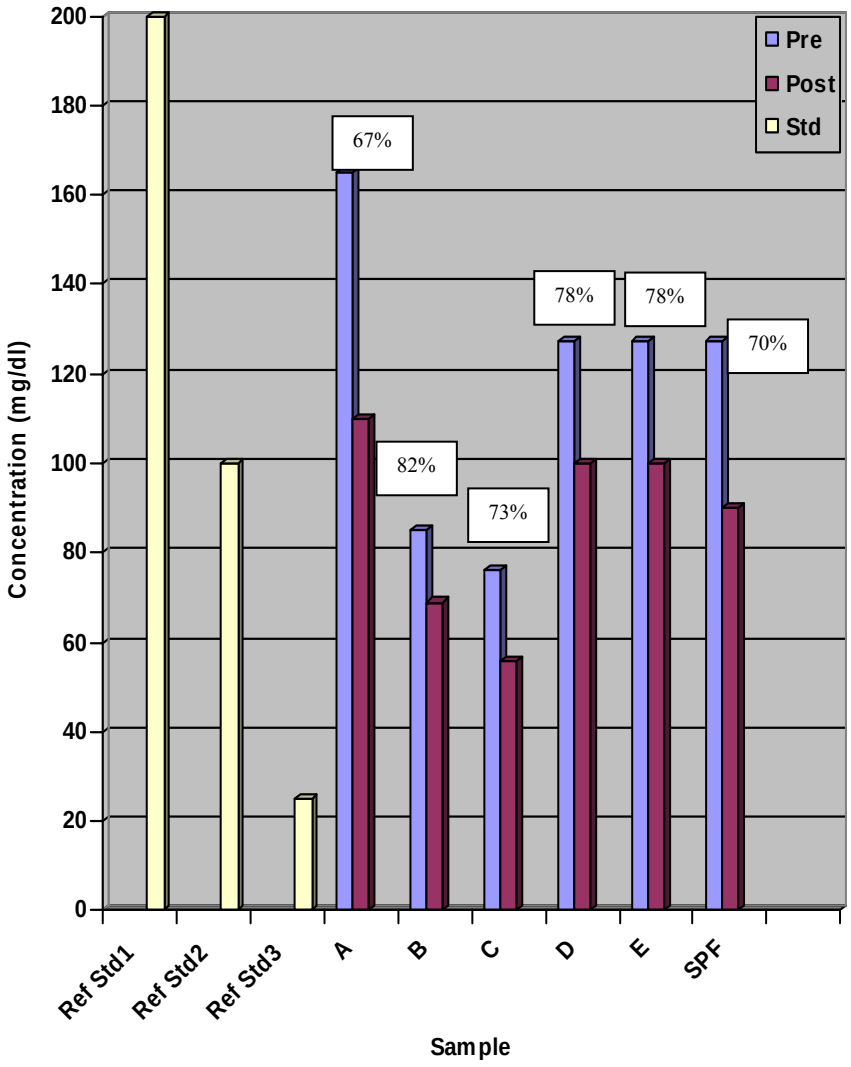


Figure 2-7. IgG concentrations of purified antibody preparations from Passive-Transfer Study 3. The IgG concentrations of pre-purification pooled serum (Pre) and post-purification antibody preparations (Post) were determined by commercial feline IgG RID assay. The IgG concentration for pooled pre-serum (grey bar) and purified antibody preparations (dark grey bar) from Fel-O-Vax FIV (Sample A), HIV-1_{UCD1} p24 (Sample B), HIV-1_{N20} p24 (Sample C), FIV_{Pet} p24 (Sample D), and FeT-J cell (Sample E) vaccinated cats and non-vaccinated SPF cats (Sample SPF) are shown with values from reference standards. Commercial reference standards corresponded to 25 (Ref Std3), 100 (Ref Std2), and 200 (Ref Std1) mg/dL. The % recovery for each purified antibody preparations is shown above the corresponding bar. The average IgG recovery was 75%, indicating an average loss of 25%.

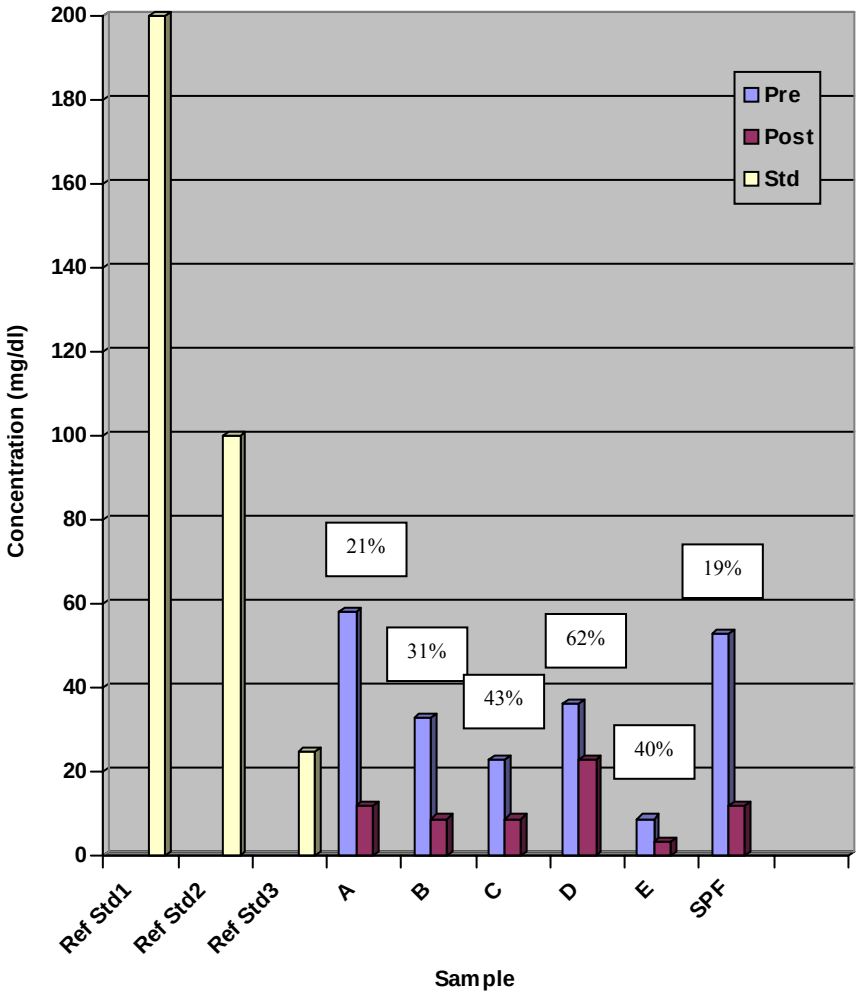


Figure 2-8. IgA concentrations of purified antibody preparations from Passive-Transfer Study 3. The IgG concentrations of pre-purification pooled serum (Pre) and post-purification antibody preparations (Post) were determined by commercial feline IgG RID assay. The IgG concentration for pooled pre-serum (grey bar) and purified antibody preparations (dark grey bar) from Fel-O-Vax FIV (Sample A), HIV-1_{UCD1} p24 (Sample B), HIV-1_{N20} p24 (Sample C), FIV_{Pet} p24 (Sample D), and FeT-J cell (Sample E) vaccinated cats and non-vaccinated SPF cats (Sample SPF) are shown with values from reference standards. Commercial reference standards corresponded to 25 (Ref Std3), 100 (Ref Std2), and 200 (Ref Std1) mg/dL. The % recovery for each purified antibody preparations is shown above the corresponding bar. The average IgA recovery was 36%, indicating an average loss of 64%.

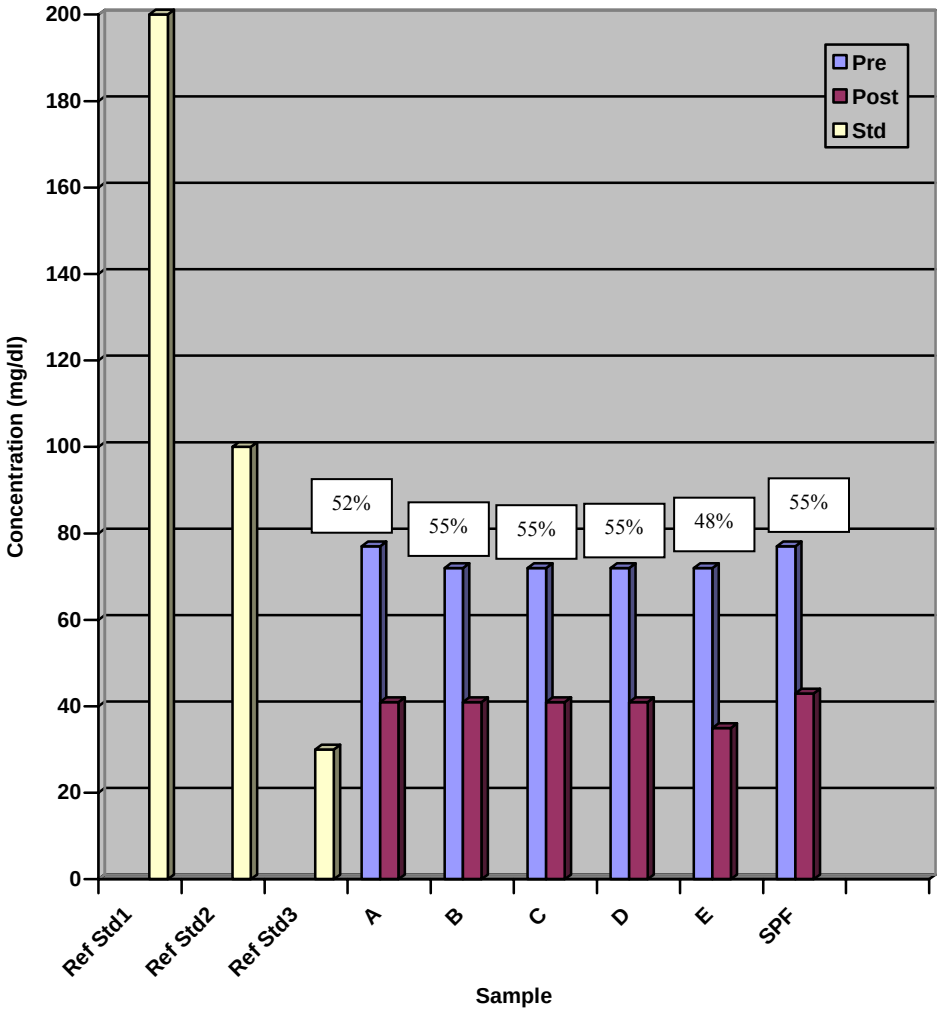


Figure 2-9. IgM concentrations of purified antibody preparations from Passive-Transfer Study 3. The IgG concentrations of pre-purification pooled serum (Pre) and post-purification antibody preparations (Post) were determined by commercial feline IgG RID assay. The IgG concentration for pooled pre-serum (grey bar) and purified antibody preparations (dark grey bar) from Fel-O-Vax FIV (Sample A), HIV-1_{UCD1} p24 (Sample B), HIV-1_{N20} p24 (Sample C), FIV_{Pet} p24 (Sample D), and FeT-J cell (Sample E) vaccinated cats and non-vaccinated SPF cats (Sample SPF) are shown with values from reference standards. Commercial reference standards corresponded to 25 (Ref Std3), 100 (Ref Std2), and 200 (Ref Std1) mg/dL. The % recovery for each purified antibody preparations is shown above the corresponding bar. The % recovery for each purified antibody preparations is shown above the corresponding bar. The average IgM recovery was 53%, indicating an average loss of 47%.

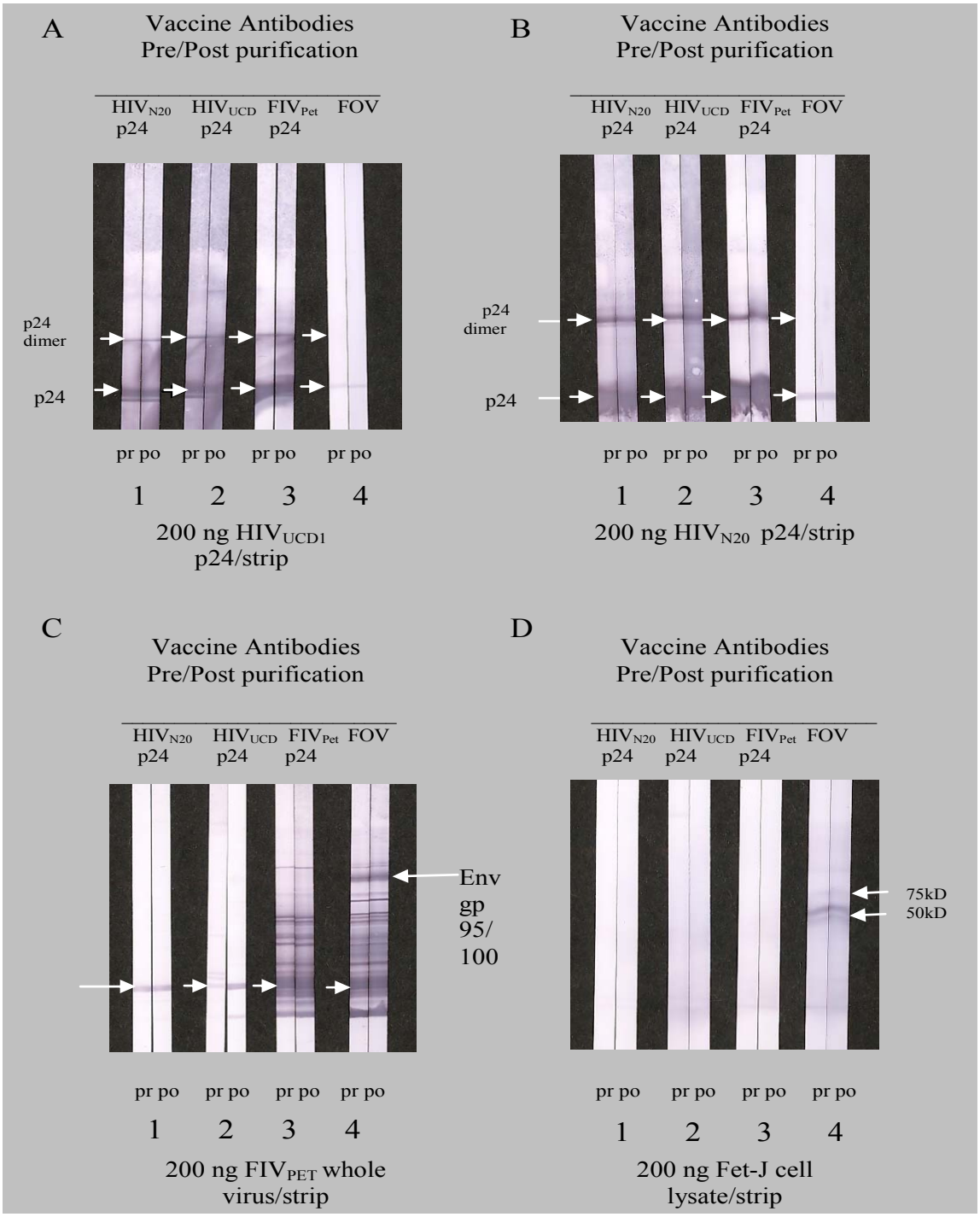


Figure 2-10. HIV-1 and FIV p24 reactivity of purified antibody preparations from Passive-Transfer Study 3. The p24 reactivity was based on immunoblot analysis using HIV-1_{UCD1} p24 (A), HIV-1_{N20} p24 (B), FIV_{Pet} whole-virus (C), and uninfected FeT-J cell-lysate (D) as substrates at 200 ng/strip. Pre-purification serum (pr) and post-purification antibody preparation (po) from HIV-1_{N20} p24 (Pre/Post Strip Pair 1), HIV-1_{UCD1} p24 (Pair 2), FIV p24 (Pair 3), and Fel-O-Vax FIV (FOV, Pair 4) vaccinated cats sera were reacted at a dilution of 1:200.

CHAPTER 3
SUBTYPE A HIV-1 P24 AS AN FIV VACCINE IMMUNOGEN

Introduction

One of the major hurdles impeding the development of an effective HIV vaccine is the necessity to elicit an immune response, which protects against different subtypes of the virus [58]. Thus far, our work on the development of an improved FIV vaccine shows that this is an achievable goal. With an aim to increase the efficacy of the FIV vaccine, a combination of two virus strains isolated from LTNP cats was utilized [59,60]. The vaccine was able to induce from moderate to significant levels of protection among SPF cats challenged with homologous and heterologous FIV strains. The vaccine induced many antibodies, which were also reactive to HIV-1 p24. The induction of these cross-reactive antibodies was expected, since several p24 antigens are conserved in the same viral subfamily. The principle of cross-protective epitopes as the basis for vaccine design has been utilized previously in the development of smallpox and canine distemper vaccines [61,62]. The studies described in this section will utilize this principle to develop an FIV vaccine that is effective against strains from multiple FIV subtypes.

Although HIV-1 and FIV p24 proteins have only 31% aa identity, core p24 of these viruses have a number of cross-reactive epitopes [38]. Previous studies from our laboratory tested the use of subtype-B HIV-1_{UCD1} p24 as an immunogen for FIV vaccines. This vaccine conferred protection in 60%-100% of vaccinated cats against strains from multiple FIV subtypes [38]. Based on this observation, current studies were performed to test the following hypothesis. *HIV-1 p24 has epitopes that are highly immunogenic to cats and some of these immunogenic epitopes that are common to HIV-1 and FIV can serve as conserved vaccine epitopes for protection of domestic cats against FIV.* In previous studies, the p24 proteins from two HIV-1 subtype-B strains had cross-protective epitopes, but such epitopes have yet to be identified on p24 from

other HIV-1 subtypes. Therefore, current studies were performed to evaluate the prevalence of cross-protective p24 epitopes on HIV-1 subtype-A. The studies included the sequencing, production, and purification of HIV-1 subtype-A p24; T-cell epitope mapping; immunogenicity analysis; and pilot vaccine-efficacy trials.

HIV-1 subtype-A p24 was sequenced from a subtype-A strain obtained from AIDS Reagent Bank. Upon confirmation of its subtype-A origin, its sequence was compared to HIV-1 subtype-B p24 (Figure 3-1) and FIV_{FC1} p24 sequences to identify potential differences in TH/CTL epitopes. Since there was no T-cell epitope database for FIV, LANL database for TH and CTL epitopes on subtype-B HIV-1 p24 was used to identify aa differences in T-cell epitopes between subtype-A and subtype-B HIV-1 p24 proteins. Vaccinated cats received 3 doses of vaccine at 3-week intervals before FIV challenge at 3 weeks after the last vaccination (Figure 3-2). These animals were challenged IV with FIV_{FC1} (Table 3-1 and 3-2).

SPF cats were immunized with either subtype-A or subtype-B HIV-1 p24 protein and their PBMC were assessed for T-cell responses to FIV and HIV-1. The interferon-gamma (IFNγ) ELISpot analysis with overlapping FIV p24 peptides was used to identify the cross-reactive T-cell epitopes. Results showing similar epitope recognition between the two vaccinated groups will indicate strong potential for subtype-A p24 vaccine to work as effectively as subtype-B HIV-1 p24 vaccine. However, results showing disparity in the T-cell epitope responses will suggest potential difference in vaccine efficacy between these two vaccines. Thus, these analyses were used to project the outcome of subsequent vaccine-efficacy trials against subtype-B pathogenic FIV_{FC1}.

Since the previous report had only one HIV-1 p24 vaccine study using FIV_{FC1} as challenge virus, the goals of Vaccination Study 1 were to test the reproducibility of the original findings

and to determine the level of vaccine protection observed in the vaccinated donors for Passive-Immunity Study 1 in Chapter 2. The vaccination groups in Vaccination Study 1 consisted of subtype-B HIV-1_{UCD1} p24-vaccinated cats, Fel-O-Vax FIV-vaccinated cats, and age-matched non-vaccinated cats. Upon conformation of protection with HIV-1 subtype-A p24 vaccine against $FIV_{FC1,}$ Vaccination Study 2 was initiated with vaccinated donors from Passive-Immunity Study 3 (Chapter 2). The vaccination groups consisted of subtype-A HIV-1_{N20} p24, subtype-B HIV-1_{UCD1} p24-vaccinated cats, FIV_{Pet} p24-vaccinated cats, Fel-O-Vax FIV-vaccinated cats, and age-matched non-vaccinated cats.

Overall, these vaccination studies were performed to determine the prevalence of cross-protective epitopes on HIV-1 subtype-A p24 and to test the hypothesis on cross-reactive epitopes for vaccine prophylaxis. Furthermore, these studies were the first step towards our central goal to provide insight into the development of an HIV-1 vaccine for humans.

Methods

Virus Selection

HIV-1 subtype-A isolates were obtained from AIDS Reagent Bank and stored in a secure freezer at -80^{o}C. The isolates were amplified by cell culture and then sequenced to identify the strain to be used as the p24 immunogen for subtype-A vaccine.

Co-culturing for Virus Amplification

Whole blood was collected from a healthy human donor, and PBMCs isolated using the ficoll-hypaque gradient technique. These cells were resuspended in culture media (RPMI 1640, FBS 10%, Gentomycin, and 2-mecaptoethanol) and stimulated with 0.1µg/mL staphylococcal enterotoxin A for 3 days. The cells were then ready to be co-cultured with HIV-1 infected cells. Cells were cultured at 3 day intervals by removing two-third of the culture media and replacing it with fresh culture media. The supernatant from each flask was stored for RT assay analysis. The

cells were monitored daily to observe changes in morphology or the formation of multinucleated giant cells indicative of HIV-1 infection. When the cultures were observed to contain a significant amount of these large multinucleated cells, they were terminated to harvest the proviral DNA.

Isolation of Proviral DNA

The cell suspension from the HIV co-cultures were placed into 15-mL tubes and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and the cells were washed in PBS. The cell pellet was resuspended in 200 µL lysis buffer (0.45%Tween 20, 0.45% NP40, PCR Buffer, 200 µg/mL proteinase K) and incubated at 56^{o}C for 24 hours. After heat treatment, an equal volume of phenol-chloroform-isomyl alcohol was added and the suspension gently rocked for 4-18 hours. The sample was subsequently votexed followed by centrifugation at 13000 rpm for 5 minutes. The upper layer containing the proviral DNA was transferred to a new microfuge tube and mixed with 50 µl of 3M sodium acetate and 400 µL of ethyl alcohol. The supernatant was removed and the pellet was rinsed in 1 mL of 70% ethanol. The sample was then centrifuged, and the supernatant removed to recover purified DNA. The DNA recovered was resuspended in DNase free H_2O and stored at -80^{o}C until its use in PCR amplification for proviral p24 sequencing.

Sequencing and Expression of HIV P24

PCR primers were designed based on the consensus and published sequences and reacted with the regions upstream and downstream of the p24 gene of HIV-1 (Figure 3-3). PCR conditions were optimized, and the following condition was used: the initial denaturing step at 94^{o}C for 2 minutes followed by 30 cycles of 30 seconds at 94^{o}C, 1 minute at 43^{o}C (*gag*) or 52^{o}C (*p24*), additional 1 minute at 72^{o}C, and the final denaturation step of 10 minutes at 72^{o}C. The reaction was performed in several 25-µL PCR reactions. The products of these reactions were

pooled and run on an 0.8% tris acetate EDTA gel electrophoresis for 15 minutes to separate the target band from primer dimers and other contaminating non-target nucleic acids (Figure 3-4). This band was then carefully excised from the gel, and the product was purified with a Qiagen gel purification kit. The purified product was measured (concentration of DNA/protein) and set to 0.5 µg/µL, and then sent to ICBR Sequencing Laboratory with accompanying primers. This procedure was performed 3X from start to finish ensuring the accuracy of the sequence result.

Both HIV-1 p24 and FIV p24 protein were expressed by *E. coli* M15 cells that were transfected with recombinant construct of lentiviral p24 and pQE 30 expression vector (Figure 3-5). This expression system produced p24 tagged with a 6X histidine residue chain. The tag was utilized in the protein purification.

Altered primer pairs were designed with adaptors on both ends of the gene insert to facilitate restriction enzyme digestion of the ends of the PCR product and to allow sticky-end ligation of the target gene in the correct orientation to the promoter of the vector. The histidine tag was placed on the N-terminus of the protein product. The endotoxin levels for each p24 protein preparation were measured using LAL E-toxate assay (Sigma Aldrich USA). All purified proteins were below 50 endotoxin units/dose, which is the endotoxin dose previously tested to be safe for use in cats.

HIV-1_{N20} primers

Two pairs of primers were used for sequencing of the whole *gag* and *p24* regions of the proviral genome. The whole *gag* sequence primer pair consisted of the forward primer HGagF (5'- GGACTCGGCTTGCTGAAGCGCGC-3') and reverse primer HGagR (5'-ATCATCTGCTCCTGTATCTAATAG-3') (Figure 2). The p24 primer pair consisted of forward primer HG1 (5'-CAGCATTATCAGAAGGAGCCAC-3') and reverse primer HG2 (5'-CACTCCCTGACATGCTGTCATCAT-3'). Using the PCR conditions mentioned earlier, the

Gag primer pair produced a fragment that was 1.685 kB long, while the p24 primer produced a fragment 542 bp long, which was within the *p24* gene. Alternate reactions with HGagF/HG2 and HG1/HGagR produced fragments of 1.115 kB and 1050 bp, respectively.

The primers used for expression were designed to introduce restriction enzyme sites (RES). The sequences for forward and reverse primers were 5'CGCG/GATCCGCGCCTATAGTGCAAAATGCA-3'and 5'-CCCA/AGCTTGGGCTACAAAACCCTTGCTTTATG-3', respectively. The underlined bases correspond to the cassette and RE integration sites in the primer (Figure 3-6).

Restriction enzyme digestion

The restriction enzyme reaction using Buffer E was designed to facilitate the digestion of both the insert DNA and the plasmid DNA, and the simultaneous digestion of both enzymatic sites with high fidelity. The digestion reaction of the pQE 30 vector contained 6 µL plasmid DNA, 0.5 µL Hind III, 0.5 µL BamH1, 1 µL Buffer E, 1 µL RNase A, and 1 µL 10X BSA. The digestion reaction of the HIV_{N20} p24 insert contained 3 µL plasmid DNA, 0.5 µL Hind III, 0.5 µL BamH1, 1 µL Buffer(E), 1 µL RNase A, and 1 µL 10x BSA. Both reactions were performed at 37^{o}C for 3 hours.

Expression and purification of p24

The expression system utilized to produce recombinant HIV-1 p24 protein was the pQE-30 expression vector transfected into chemically competent *E. coli* M15 cells (derived from *E. coli* K12). This expression system produced p24 protein with a 6X histidine tag, which was used later for purification. Altered primer pairs were designed to introduce adaptors on both ends for facilitating restriction enzyme digestion of the ends of the PCR product and to allow sticky end ligation of the target gene in the correct orientation to the promoter sequence. The histidine tag was placed on the N-terminus of the protein product. The 6X histidine tag facilitated the binding

of the recombinantly expressed p24 protein to a Ni-NTA resin used in affinity purification to remove contaminating *E. coli* proteins. M15 cells were transformed with vector plus insert under standard conditions (Sambrook *et al.* 1989) and plated on selective media containing 25 µg/mL of kanamycin and 100 µg/mL of ampicillin. After overnight incubation at 37°C, the colonies grown on the replica plate were screened by colony-direct PCR to ensure that they contained the target insert. After screening, glycerol stock was prepared from confirmed colonies and stored at -80°C. Two flasks, both containing 20 mL Lauria Bertani (LB) broth with 100 µg/ml of ampicillin and 25 µg/mL of kanamycin were inoculated with the selected M15 colony and cultured overnight at 37°C. Culture flasks, each containing 500 mL LB broth (100 µg/mL of ampicillin and 25 µg/mL of kanamycin), were inoculated with 1:50 dilution of the overnight culture. The cells were allowed to grow for approximately 2.5 hours, until an optical density of 0.6 was reached. The culture was then induced using IPTG at a final concentration of 1mM. Throughout 4.5 hours of culturing, 1ml of the sample was collected for analysis. The cultures were then centrifuged at 4000g for 20 minutes, and the pelleted cells stored overnight at -20°C.

Purification

E. coli pellets were thawed on ice, then re-suspend in Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) (3 mL/g *E. coli*). The cells were then sonicated to disrupt cell walls, which allowed complete lysis. Protease inhibitor was added to the lysate to inhibit the degredation of the expressed protein, and the suspension was centrigufed at 10000g for 30 minutes to remove the cellular debris. Some of supernatant was saved for analysis, and the remaining fraction was purified under native conditions using Ni-NTA affinity chromotography column according to Ni Super Flow specifications (Qiagen Inc., Valencia, California). The purified products were tested by CBB and silver stain analyses, endotoxin test, Bradford protein

assay (BioRad Systems, Inc. Hercules, California) according to manufacturers protocol and immunoblot analysis[40].

Vaccination Studies 1 and 2

SPF cats from Liberty Research (Vaccination Study 1) and Harlan Sprague Dawley, Inc. (Vaccination Study 2) were immunized 3X at 3-week intervals with either HIV-1 p24, FIV p24, or Fel-O-Vax FIV vaccine. Cats were also immunized with PBS or FeT-J cell/lysate combination (as described in Chapter 2 and detailed below). The p24 vaccines were 200 μg of p24 formulated in Ribi (Study 1) or FD-1 (Study 2) adjuvant supplemented with recombinant feline IL-12 (R&D Systems, Inc., Minnesota, MN). Fel-O-Vax FIV vaccine is a commercial product formulated in FD-1 adjuvant. FD-1 adjuvant generally provided better efficacy results than Ribi adjuvant. Study 1 consisted of HIV-1_{UCD1} p24-vaccinated (n=4), Fel-O-Vax FIV-vaccinated (n=4), and PBS-immunized (n=3) groups, and used an FIV_{FC1} challenge of 15 CID_{50}. Study 2 consisted of HIV-1_{UCD1} p24-vaccinated (n=4), HIV-1_{N20} p24-vaccinated (n=4), FIV_{Pet} p24-vaccinated (n=3), Fel-O-Vax FIV-vaccinated (n=2), and FeT-J cell-immunized (n=2) groups, and used an FIV_{FC1} challenge of 10 CID_{50}.

The FeT-J cell/lysate-immunized cats received a combined cell and cell-lysate immunization using SC ($2x10^6$ cells/dose), intradermal (ID, 250 μg/dose cell lysate), intranasal (IN, 100 μg/dose cell lysate), and transcutaneous (TC, 100 μg/dose cell lysate) routes. The total number of cells/dose for SC immunization was equivalent to the inactivated infected cells ($2x10^6$ cells/dose) and viral proteins (50 μg/dose) in the Fel-O-Vax FIV vaccine. The total cell-lysate dose for combined ID/IN/TC/SC immunization was 500 μg/dose. This amount was equivalent to the amount of inactivated dual-subtype whole-viruses in prototype vaccines, which also contained a moderate level of cellular debris. Therefore, this cell-lysate dose can be used safely

in cats. This high dose was close to 2X the amount of p24 proteins and was 10X the whole-viral proteins in Fel-O-Vax FIV vaccine. This high cell-lysate dose in combination with uninfected cells was used to ensure that sufficient amount of MHC was in the preparation to serve as cell control for Fel-O-Vax FIV vaccine. FIV-infected FeT-J cells have higher levels of MHC expressed on the cells than uninfected FeT-J cells. Furthermore, the FIV virion, much like HIV-1, has a concentrated amount of host's MHC on the viral membrane. Previous inactivated SIV vaccine studies in macaques demonstrated protection against SIV with uninfected human cells used to grow the vaccine virus. In this study, the xenogeneic MHC of the human cells present in the inactivated SIV vaccine provided the protection against SIV challenge, which was also grown in human cells.

Feline IFNγ (Fe IFNγ) ELISpot analysis

Commercial FeIFNγ ELISpot (R&D Systems, Inc.) was used to determine HIV-1 p24 and FIV p24 peptide responses of the PBMC from the HIV-1_{UCD1} p24-, HIV-$_{1N20}$ p24-, FIV p24-, and Fel-O-Vax FIV-vaccinated cats. Fresh cells ($2x10^5$ cells/well) suspended in feline assay media were cultured with peptides at 1 μg peptide/well in FeIFNγ ELISpot plate for 18 hours. FeIFNγ ELISpot plates were processed according to manufacturer's methods (R&D Systems, Inc.) with the exception of the assay media used, and analyzed with an ELISpot reader. Feline assay media consisted of AIM-V media (Gibco-Invitrogen Co., Carlsbad, CA), 10% heat-inactivated pooled SPF sera, and 25 μg/ml gentamycin (Mediatech, Inc., Herndon, VA). All assays included T-cell mitogen, concanavalin A (Con A) (Sigma-Aldrich USA), at 2-4 μg/well as the positive control; recombinant HIV-1_{UCD1}, HIV-1_{N20}, and FIV_{Pet} p24 proteins and inactivated FIV_{Pet} plus FIV_{Shi} whole-viruses at 2 μg/well as virus-specific immunogens; and FIV_{Bang} p24 peptide F8 at 5 μg/well as the negative control. In addition, LPS at 5 and 50

EU/well was included as a control for recombinant products. The LPS used for control was from *E. coli* M15, which was used to produce the recombinant p24 proteins. These products had 1.19 EU per ng, whereas the recombinant vaccine had <50 EU per vaccine dose of 200 μg. All results were in duplicates and adjusted to spot forming units (SFU) per $1x10^6$ cells, after subtracting the background derived from non-specific peptide control or media control, whichever was higher in value. The standard deviation of duplicate results for the feline IFNγ ELISpot analysis was <15% of the median SFU.

Overlapping 15mer peptides for HIV-$1_{LAI/LAV}$ (identical to NIH reference subtype-B virus HIV-1_{HXB2}) with 11 aa overlap were produced by Synpep Corporation, USA). Each peptide pool was designated as HIV-1 peptide pool number 1-18 (Hp1-Hp18). Peptide-pool Hp1 consisted of the first four overlapping peptides from the amino-terminal, and each peptide pool Hp2 through Hp18 contained three consecutive overlapping peptides starting from the amino-terminal. The FIV peptides consisted of overlapping 15mer peptides for FIV_{Bang} (FIV subtype-A p24 sequence) with 11 aa overlap and also produced by (Synpep Corporation, USA). Each FIV peptide pools consisted of 3-4 consecutive peptides and were designated as FIV peptide pool number 1-17 (Fp1-Fp17).

Statistical Analyses

Individual immunization groups, including protected *versus* infected cats in each study, were analyzed for statistical significant difference by Mann-Whitney Rank Sum Test using Sigma Stat (Windows Version 3.11). The difference was considered statistically significant when $P<0.05$.

Results

Analysis of HIV-1N20 P24 for Vaccine

Silver-stained PAGE gel (Figure 3-7) and CBB-stained gel (data not shown) analyses showed minimal contamination and a purity of 95% using BSA standard to extrapolate the p24 concentration. The silver-stained gel showed three bands at 24 kD (62.5-500 ng lanes), 20 kD (250-500 ng lanes), and 48 kD (500 ng lane) (panel C). The HIV-1 p24 specificity of the 24 kD product and truncated products was detected by immunoblot analysis using sheep anti-HIV-1 p24 antibodies (Cliniqa Corporation, Fallbrook, California) (panel A). The HIV-1 p24 bands at 24 kD were detectable from 31.25-500 ng, and a higher band at 48 kD at 500 ng appeared to be p24 dimer. The two lower bands in the immunoblot at 15 kD and 20 kD were observed at 62.5-500 ng and 125-500 ng, respectively, and appeared to be truncated p24 products (panel A). The level of endotoxin in the HIV-1_{N20} p24 preparation used for vaccine was 1.19 EU/ng (2.38 EU/dose of vaccine), which was safe level for use as vaccine (LPS safety level previously tested as <50 EU/dose).

HIV-1 and FIV Sequence Analyses

Sequences comparison of FIV_{FC1} p24 Challenge virus) with HIV-1_{N20} and HIV-1_{UCD1} p24 revealed the challenge virus to possess 52.1% homology and 31.3% identity with HIV-1_{UCD1} p24 compared to 50.8% homology and 30.5% identity with HIV-1_{N20} p24. The HIV-1 p24 sequence is 231 aa long compared to 223 of FIV p24. This resulted in alignment gap generation of 6.0% and 7.6% gaps for HIV-1_{UCD1} and HIV-1_{N20} p24, respectively. HIV-1_{N20} p24 contained aa substitutions compared to HIV-1_{UCD1} p24 within the 3 of the 4 published p24 CTL epitopes shown on Figure 3-1, with a P → S and I → M substitution in the first epitope shown, a K to R substitution in the third and a S T→ and D →E substitution in the fourth. The second epitope was identical between the two sequences.

Vaccination Studies 1 and 2

In Vaccination Study 1 (Table 3-1), 3 of 4 $HIV1_{UCD1}$ p24-vaccinated cats (Group 1A) and 4 of 4 Fel-O-Vax FIV-vaccinated cats (Group 1B) were protected against FIV_{FC1} challenge (15 CID_{50}), which infected all three PBS-immunized control cats (Group 1C). The unprotected/vaccinated cat (#AA1) and infected control cats had a major decrease in $CD4^+$-cell (CD4) counts and $CD4^+$-cell/$CD8^+$-cell (CD4/CD8) ratio when compared to the values from protected/vaccinated cats. A comparison between infected cats and protected/vaccinated cats at 20 wpi showed only statistically significant difference for CD4/CD8 ratio (CD4 count, p=0.14; CD4/CD8 ratio, p=0.001). However, statistical difference was observed between the mean CD4/CD8 ratios of Fel-O-Vax FIV-vaccinated group and PBS-immunized control group at 20 wpi. These results demonstrate immune protection in the protected/vaccinated cats and further support the results from FIV antibody and virus isolation analyses that these cats were clearly not infected.

In Vaccination Study 2 (Table 3-2), 2 of 4 $HIV1_{UCD1}$ p24-vaccinated cats (Group 2A), 1 of 4 $HIV1_{N20}$ p24-vaccinated cats (Group 2B), and 0 of 3 FIV p24-vaccinated cats (Group 2C) were protected against FIV_{FC1} challenge (10 CID_{50}). All Fel-O-Vax FIV-vaccinated cats (Group 2D, n=2) were also protected, while all control cats immunized with FeT-J cell/lysate (Group 2E, n=2) were infected. Overall, the protected/vaccinated cats had higher CD4 counts and CD4/CD8 ratios than unprotected/vaccinated cats and infected control cats. In fact, statistical differences in both the mean CD4 counts (p=0.019) and the mean CD4/CD8 ratios (p<0.001) were observed between protected/vaccinated cats and infected cats. These statistical differences were due to the infection and were not due to the difference in the mean CD4 counts of the pre-challenge groups (Table 3-2; Groups A *vs.* B *vs.* C *vs.* D *vs.* E: CD4 count, p>0.10; CD4/CD8 ratio, p>0.2). This pilot study confirms the results from Vaccination Study 1 that $HIV1_{UCD1}$ p24 vaccine and Fel-O-

Vax FIV vaccine are effective against FIV_{FC1}. In contrast, minimal protection was achieved by $HIV1_{N20}$ p24 vaccination. The level of protection (2 of 4) achieved with HIV_{UCD1} vaccine in Vaccination Study 1 was slightly lower than Vaccination Study 2 (3 of 4) even though the challenge dose in Study 1 was slightly higher than Study 2.

IFNγ ELISpot Analysis of PBMC from Vaccination Study 2

As a measure for vaccine-induced cellular immunity, FIV-specific IFNγ responses of PBMC from vaccinated cats were compared. PBMC from HIV-1_{UCD1} p24-vaccinated cats had more cats responding to overlapping HIV-$1_{LAI/LAV}$ p24 and FIV p24 peptides (Hp3, Hp11, Hp12; Fp9) than PBMC from HIV-1_{N20} p24-vaccinated cats (Figures 3-8, and 3-9, Panels A and B). However, the PBMC from both HIV-1_{UCD1} p24- and HIV-1_{N20} p24-vaccinated cats (2 of 4 cats from each group) responded to dual-subtype FIV whole-virus (IWV) immunogen. These results clearly indicated that the PBMC from HIV-1 p24-vaccinated cats recognized cross-reactive epitopes on FIV p24. Two HIV-1 p24-vaccinated cats also recognized Fp5 although one cat had response slightly below the threshold value (figure 3-9A). The PBMC of all protected cats from the HIV-1 p24-vaccinated groups (#185, #205, #207) responded to Fp9 peptide pool but the response from cat #207 was way below the threshold level of significance. Moreover, the PBMC from Fel-O-Vax FIV-vaccinated cat #171 and FIV p24-vaccinated cat #163 had a significant cross-reactive response to Hp11 (Figure 3-8C). Hence, two-way cross-reactivity was observed, whereby HIV-1 p24-vaccinated cats recognized Fp9 epitope, while Fel-O-Vax FIV- and FIV p24-vaccinated cats recognized Hp11.

The PBMC from the two Fel-O-Vax FIV-vaccinated cats had robust IFNγ response (Figure 3-9C). One cat (#171) had significant responses ($\geq$50 SFU threshold) to a number of FIV p24 peptide pools, including Fp9, but the other protected cat (#183) had no response to Fp9 (Figure

3-9A, C). Both cats had a significant response to Fp3, suggesting the potential of multiple epitopes for protection. Thus, 4 of 5 protected cats vaccinated with HIV-1 p24 or Fel-O-Vax FIV vaccine reacted to Fp9, of which 3 responses were above the threshold response, while none of the unprotected/vaccinated cats had significant response to Fp9 (Figure 3-9A, B and C). In any event, the numbers of cats are much too limited in the current study to determine the cross-protective epitope(s) solely based on one parameter (i.e., IFNγ response) of cellular immune response.

Discussion

The efficacy of Vaccination Studies 1 and 2 are summarized in Table 3-2 along with results from other published and unpublished studies of our laboratory. The combined results from current two studies show subtype-B HIV-1_{UCD1} p24 vaccine conferring the highest protection rate of 62% (5 of 8 cats) among the two HIV-1 p24 vaccines tested against FIV_{FC1} strain. Only minimal efficacy (1 of 4) was observed with subtype-A HIV-1_{N20} p24 vaccine in Vaccination Study 2. This observation was somewhat in conflict with the sequence results since considerable sequence identity and homology existed between HIV-1_{N20} and HIV-1_{UCD1} p24 sequences. However, the numbers of animals used in each group were much too small to make major conclusion. In fact, the efficacy of HIV-1_{UCD1} p24 in Study 2 had the lowest rate, along with another study, among the seven vaccination studies performed so far (Table 3-2). Since the efficacy was also low for HIV-1_{UCD1} p24 vaccine in Study 2, more studies will be needed to determine if significant difference in efficacy exists between subtype-B HIV-1_{UCD1} p24 vaccine and subtype-A HIV-1_{N20} p24 vaccine.

One potential reason for lower efficacy in Study 2 than Study 1 may be due to the SPF cats used. SPF cats from Liberty Research and Harlan Sprague Dawley, Inc. were used in Studies 1

and 2, respectively. In general, cats from Harlan Sprague Dawley. were more susceptible to FIV infection and more difficult to confer protection with HIV-1 p24 and Fel-O-Vax FIV vaccines than cats from Liberty Research (R. Pu and J.K. Yamamoto, personal communiqué). Based on MHC-I sequence analysis of one cat from Harlan Sprague Dawley and our semi-inbred cats derived from backcross inbreeding with tom from Liberty Research, cats from Harlan Sprague Dawley had MHC-I sequence lineage different from the predominant lineages of the cats from Liberty Research (E. Sato and J.K. Yamamoto, personal communiqué). Furthermore, according to our findings from Chapter 2, both HIV-1 p24 and Fel-O-Vax FIV vaccines conferred protection against FIV_{FC1} by cellular immunity. Recent adoptive-transfer studies by our laboratory demonstrated protection with prototype dual-subtype FIV vaccine was mediated by $CD4^+$ CTL and $CD8^+$ CTL [57]. Hence, MHC-restricted T-cell immunity may play a central role in protection observed with HIV-1 p24 vaccine.

The importance of cellular immunity was determined in current studies by monitoring the FIV-specific IFNγ responses of the vaccinated cats. HIV-1_{UCD1} p24 vaccine was more immunogenic than HIV-1_{N20} p24 vaccine, but not as immunogenic as Fel-O-Vax FIV vaccine. In current study, only 1 of 3 cats vaccinated with FIV_{Pet} p24 had robust IFNγ responses (Fp7, Fp8, Fp14, Fp15), while another cat (#195) from the same group had a single significant response to Fp3. The PBMC from both Fel-O-Vax FIV-vaccinated cats had significant responses to two or more FIV peptide pools. Thus, Fel-O-Vax FIV vaccine appeared to be more immunogenic than FIV p24 vaccine. This observation supported the previous study of our laboratory, which showed only 2 of 7 FIV p24-vaccinated cats with low but above the threshold responses to FIV p24 peptide pools (Fp3, Fp7, and Fp10). Fp3 and Fp7 were the common FIV

peptide pools recognized by the cats from both studies. Furthermore, all cats, which responded to these peptide epitopes, were from the same vendor (Harlan Sprague Dawley).

The PBMC from HIV-1_{UCD1} p24-vaccinated cats had IFNγ responses to Hp3 (2 of 4), Hp11 (3 of 4), and Hp12 (4 of 4), while the PBMC from Fel-O-Vax FIV-vaccinated cats and FIV p24-vaccinated cats had a significant response to Hp11 (2 of 5). In the previous unpublished studies of our laboratory, the PBMC from HIV-1_{UCD1} and HIV-$1_{LAI/LAV}$ p24-vaccinated cats had significant responses to Hp3 (1 of 3), Hp6 (4 of 8), Hp11 (6 of 8), Hp12 (2 of 8), Hp14 (3 of 8), and Hp17 (2 of 8). Current study had lower numbers of peptide epitopes recognized by the HIV-1 p24-vaccinated cats than the previous studies. This may be due to the more diverse MHC patterns present in the cats from previous studies, since previous studies used three sources of cat vendors. Only 2 of the 8 cats were from the same vendor as current study. Moreover, the one cat that responded significantly to all Hp3, Hp11, and Hp12 in the previous study was from the same vendor as the current study. Whereas, the other cat from the same vendor in previous study responded slightly below the threshold value to Hp11 and Hp12, but had no responses to other peptide epitopes. In current study, the PBMC from the two Fel-O-Vax FIV-vaccinated cats had a significant response to Fp3. In the previous study, the PBMC from 8 of 8 cats vaccinated with prototype vaccine of Fel-O-Vax FIV vaccine, had significant responses to Fp3. Thus, Fp3 may be the immunodominant epitope for both prototype and commercial vaccines. Other major epitopes recognized by prototype vaccine were Fp7, Fp10, and Fp13 (5 of 8 each) followed by Fp1 (3 of 8), Fp2 (4 of 8), Fp8 (3 of 8) Fp9 (3 of 8), Fp11 (3 of 8), and Fp15 (4 of 8). Thus, both prototype and commercial vaccines induced strong IFNγ responses to multiple epitopes. Findings from recent adoptive-transfer studies from our laboratory [7] further support the importance of MHC-restricted cellular immunity in prototype vaccine of the commercial

vaccine. These findings taken together with results from HIV-1 p24 vaccine studies and passive-transfer studies in Chapter 2 suggest the importance of MHC-restricted cellular immunity in protection conferred by subtype-B HIV-1 p24 vaccines.

FIV_{Pet} 24 vaccine conferred no protection (0 of 3) in Vaccination Study 2. This result taken together with other FIV p24 studies demonstrated a very minimal protection with FIV p24 vaccine (Table 3-2, 3 of 21, 14%). Moreover, subtype-B HIV-1 p24 vaccines were far more effective against FIV challenge than FIV p24 vaccine (64-67% *versus* 14%, Table 3-3). A comparison of HIV-1_{UCD1} and FIV_{FC1} p24 sequences demonstrated only 31.3% identity and 52.1% homology between them. Two potential explanations can be provided. FIV p24 may possess immunodominant epitopes, which restrict the development of immune responses to protective epitopes. Protective epitopes need to stimulate functional activity essential for vaccine protection such as CTL activity to viral antigen. Many CTL epitopes have been identified on HIV-1 p24, including those that are immunodominant [LANL]. However, these CTL epitopes were identified by analyzing the CTL responses to HIV-1 p24 peptides made by the PBMC from HIV-1-positive human subjects. Since cats may not recognize the same epitopes as human, our IFNγ ELISpot analysis with overlapping FIV and HIV-1 p24 peptides were the first step towards identifying cross-protective CTL and TH epitopes induced by effecticacious vaccines (HIV-1_{UCD1} p24 and Fel-O-Vax FIV vaccines). The other possibility is that cellular response elicited by HIV-1 p24 is not reacting to the cross-reactive epitopes on the FIV p24 but instead reacting to the mimotopes elsewhere on the FIV virus. One way to examine this possibility was by performing NCBI Blast search on both homologous and divergent segments of the HIV-1_{UCD1} p24 sequence against other structural and enzymatic proteins (Env,

Pol, MA) of FIV. Initial analysis suggests no significant mimicry of p24 aa sequences elsewhere on the virus.

This study is the first to simultaneously examine the cross-protective epitopes of FIV/HIV by IFNγ ELISpot. The results seem to indicate that there is cross-recognition of epitopes with HIV-1 p24-vaccinated cats responding to FIV IWV immunogen and FIV p24 epitope Fp9. Furthermore, both Fel-O-Vax FIV-vaccinated cat #171 and FIV p24-vaccinated cat #163 responded to HIV-1_{UCD1} p24 protein and HIV-1 p24 epitope Hp11. Moreover, 3 of 4 HIV-1_{UCD1} p24-vaccinated cats responded to Hp11, with the response of the fourth cat near the threshold. Overall, these results demonstrated two-way cross-reactivity and also confirmed the existence of conserved epitopes between subtype-B HIV-1 p24 and FIV p24.

Though HIV-1_{N20} and HIV-1_{UCD1} p24 sequences are 90.5% identical and 95.2% homologous, there was a considerable difference in the protection rate of the two groups with 62% protection for HIV-1_{UCD1} p24 vaccinated cats and only 25% efficacy for cats vaccinated with HIV-1_{N20} p24. Both vaccinated groups generated antibodies to HIV-1 p24, but the ELISpot result showed less overall reactivity of HIV-1_{N20} p24-vaccinated cats compared to HIV-1_{UCD1} p24-vaccinated cats to HIV-1 p24 peptides. Two reasons for this disparity in IFNγ responses can be speculated. This could also be the result of aa substitutions within the corresponding regions of HIV-1_{N20} p24. It is also possible that the difference in reactivity could be based on the fact that the peptide pool was designed based on HIV-1_{HXB2} (identical to HIV-$1_{LAV/LAI}$), which is more similar to HIV-1_{UCD1} p24 than to HIV-1_{N20} p24. One reasons for selecting this sequence was because of its sequence variation within the CTL epitopes shown in Figure 3-1, which are recognized by both cats and humans. Though the substitutions were similar in charge this change

or the steric alteration of cleavage patterns by flanking non-homologous substitutions may be responsible for the disparity in the immunogenicity and the protection rate of these vaccines.

In current studies, Fel-O-Vax FIV vaccine conferred 100% protection (6 of 6). The high protection rate against heterologous-subtype FIV_{FC1} was in agreement with previous published and unpublished results (Table 3-2). These data taken together clearly demonstrate that the commercial Fel-O-Vax FIV vaccine provides considerable protection (Table 3-2, 17 of 18) against heterologous-subtype-B virus. Furthermore, long-duration vaccine analyses of Fel-O-Vax FIV vaccine showed 50-56% protection in cats challenged with FIV_{Bang} and FIV_{FC1} (both 25 CID_{50}), respectively, after 1-year boost. Since natural transmission doses are reported to be far lower than the lowest dose (10 CID_{50}) used in current studies [23,57,63], commercial Fel-O-Vax FIV vaccine is likely to be more effective against natural transmission. A pilot study performed by our laboratory also showed long-term efficacy (67%, 2 of 3) with HIV-1_{UCD1} p24 vaccine against subtype-A/B recombinant FIV_{Bang} challenge administered after 1-year boost (Table 3-2). Since single boost 1-year later was sufficient enough to provide 67% protection, the immunity elicited during the first three vaccinations must have sustained, in order for a single 1-year boost to confer such level of protection.

Table 3-1. Vaccination Study 1 with HIV-1_{UCD1} p24 vaccine and commercial Fel-O-Vax FIV vaccine

Group	Cat #	Vaccination	FIV Chal. (CID_{50}) [a]	CD4# (CD4/CD8) [b] Pre	CD4# (CD4/CD8) [b] Post	Weeks Post-Challenge (FIV Ab/VI) [c] 3	6	9	12	15	17	20	P/L/B [c]	Protection Rate (%)
A	AA1	$HIV1_{UCD1}$ p24	FC1 (15)	3.47 (3.6)	2.44 (1.4)	+/+	+/+	+/+	+/+	+/+	+/+	+/+	ND	3/4 (75%)
	MD1	$HIV1_{UCD1}$ p24	FC1 (15)	3.47 (4.9)	3.88 (2.5)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
	MG1	$HIV1_{UCD1}$ p24	FC1 (15)	3.53 (2.7)	2.90 (1.8)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
	MF3	$HIV1_{UCD1}$ p24	FC1 (15)	1.83 (3.4)	1.71 (1.7)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
				3.07 (3.6)	***2.73 (1.8)***									
B														
	AA2	Fel-O-Vax FIV	FC1 (15)	3.58 (3.4)	3.56 (2.7)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	4/4 (100%)
	MD2	Fel-O-Vax FIV	FC1 (15)	3.41 (3.8)	2.83 (2.6)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
	MG2	Fel-O-Vax FIV	FC1 (15)	3.61 (2.8)	2.92 (2.2)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
	MF4	Fel-O-Vax FIV	FC1 (15)	1.44 (2.7)	2.13 (2.1)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-/-	
				3.01 (3.2)	***2.86 (2.4)***									
C	MD3	PBS	FC1 (15)	3.23 (2.5)	2.89 (1.4)	-/-	+/-	+/+	+/+	+/+	+/+	+/+	+/+/-	0/3 (0%)
	MG5	PBS	FC1 (15)	1.99 (2.6)	1.50 (0.8)	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/-/+	
	MK4	PBS	FC1 (15)	3.03 (4.3)	1.62 (1.1)	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/-/+	
				2.75 (3.1)	***2.00 (1.1)***									

[a] FIV challenge (FIV chal.) was subtype-B FIV_{FC1} (FC1) at 15 CID_{50}.

[b] The CD4 counts (CD4#; x1000/μL) and CD4/CD8 ratios (CD4/CD8) determined at -2 (Pre) and 20 (Post) weeks. The average count and ratio are shown in bold italics for each group.

[c] FIV infection was based on the development of FIV antibodies (FIV Ab) determined by FIV-immunoblot analysis and the presence of viruses determined by virus isolation (VI) using RT and PCR analyses. Results for the samples collected at 3, 6, 9, 12, 15, 17, and 20 wpc are shown either as positive (+) or negative (-) for FIV antibodies or viruses. Virus isolation was also performed on PBMC (P), lymph node (L), and bone marrow (B) cells at 34-46 wpc. Abbreviation is not done (ND).

Table 3-2. Vaccination Study 2 with HIV-1 p24, FIV p24, and Fel-O-Vax FIV vaccines

Group	Cat #	Vaccination Status	FIV Challenge (CID_{50}) [a]	CD4# (CD4/CD8) [b] Pre	CD4# (CD4/CD8) [b] Post	Weeks Post-Challenge (FIV Abs/VI) [c] 3	6	9	12	15	17	20	Protection Rate (%)
2 A	147	$HIV1_{UCD1}$ p24	FC1 (10)	3.85 (5.59)	0.68 (1.98)	-/-	-/-	+/-	+/+	+/+	+/+	+/+	2/4 (50%)
	185	$HIV1_{UCD1}$ p24	FC1 (10)	2.39 (3.43)	1.80 (2.42)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	199	$HIV1_{UCD1}$ p24	FC1 (10)	1.19 (2.27)	1.06 (1.55)	-/-	-/-	+/-	+/+	+/+	+/+	+/+	
	205	$HIV1_{UCD1}$ p24	FC1 (10)	2.31 (3.66)	2.98 (2.25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
				2.44 (3.74)	***1.63 (2.05)***								
2 B	175	$HIV1_{N20}$ p24	FC1 (10)	0.87 (2.09)	0.40 (1.71)	-/-	-/-	+/-	+/-	+/-	+/-	+/+	1/4 (25%)
	187	$HIV1_{N20}$ p24	FC1 (10)	3.05 (4.54)	1.40 (0.88)	-/-	+/-	+/-	+/+	+/+	+/+	+/+	
	201	$HIV1_{N20}$ p24	FC1 (10)	0.89 (3.22)	1.55 (1.20)	-/-	-/-	+/-	+/+	+/+	+/+	+/+	
	207	$HIV1_{N20}$ p24	FC1 (10)	1.90 (2.72)	2.90 (2.62)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
				1.68 (3.14)	***1.56 (1.60)***								
2 C	163	FIV_{Pet} p24	FC1 (10)	1.56 (3.20)	0.56 (1.07)	-/-	+/-	+/-	+/+	+/+	+/+	+/+	0/3 (0%)
	173	FIV_{Pet} p24	FC1 (10)	1.03 (2.78)	0.76 (0.76)	-/-	+/-	+/-	+/+	+/+	+/+	+/+	
	195	FIV_{Pet} p24	FC1 (10)	1.72 (2.54)	1.76 (1.58)	-/-	-/-	-/-	+/-	+/+	+/+	+/+	
				1.44 (2.84)	***1.03 (1.14)***								
2 D	171	Fel-O-Vax FIV	FC1 (10)	1.22 (2.49)	1.09 (2.44)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	2/2 (100%)
	183	Fel-O-Vax FIV	FC1 (10)	1.86 (2.78)	2.51 (2.25)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
				1.54 (2.64)	***1.80 (2.35)***								
2 E	189	FeT-J cell lysate	FC1 (10)	1.98 (3.22)	1.31 (1.15)	-/-	-/-	+/-	+/+	+/+	+/+	+/+	0/2 (0%)
	203	FeT-J cell lysate	FC1 (10)	2.57 (3.20)	2.61 (1.60)	-/-	-/-	-/-	-/+	+/+	+/+	+/+	
				2.28 (3.21)	***1.96 (1.38)***								

[a] FIV challenge was subtype-B FIV_{FC1} (FC1) at $10CID_{50}$.

[b] The CD4 counts (CD4#; x1000/μL) and CD4/CD8 ratios (CD4/CD8) determined at -2 (Pre) and 20 (Post) weeks. The average count and ratio are shown in bold italics for each group.

[c] FIV infection was based on the development of FIV antibodies (FIV Ab) determined by FIV-immunoblot analysis and the presence of viruses determined by virus isolation (VI) using RT and PCR analyses. Results for the samples collected at 3, 6, 9, 12, 15, 17, and 20 wpc are shown either as positive (+) or negative (-) for FIV antibodies or viruses. Virus isolation was also performed on PBMC (P), lymph node (L), and bone marrow (B) cells at 34-46 wpc.

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Figure 3-1. Amino acid alignment of HIV-1_{N20} p24 (N20) with a subtype-A consensus sequence (ConsA) and with HIV-1 p24. The stars under each amino acid pair indicate identical bases. The number of dots listed below the non-identical pairs indicates the charge similarity of the amino acids with 2 dots indicating a greater similarity in charge signature than 1 dot, with no dots being indicative of least charge similarity. The alignment comparison between HIV-1_{N20} and consensus sequences indicates that HIV-1_{N20} was a good representative sequence of the subtype-A, and is not an outlier. Therefore it should contain epitopes typical of this subtype. The boxes highlight established CTL epitopes in the Los Alamos Database.

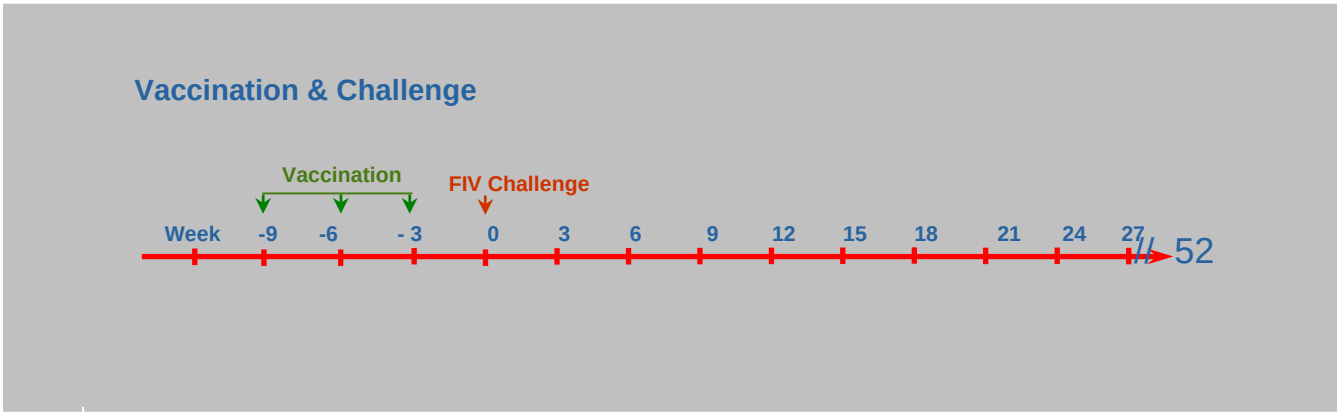


Figure 3-2. Schedule for vaccination and challenge for vaccination studies. Vaccinated cats received three of vaccine at 3-week intervals and received FIV challenge 3-weeks after the last boost. These animals were challenged IV with FIV_{FC1}.

TTCGCGCTTAACCCTAGCCTTTTAGAAACAGCGGAAGGATGTCAGCAACTAATGGAACAGTT
ACAATCAGCTCTCAAGACAGGGTCAGAAGAACTTAAATCATTGTTTAACACCATAGCAACCC
TTTGGTGCGTGCATCAAAGGATAGATGTAAAAGACACCAAGGAAGCCTTAGATAAAGTAGA
GGAAGTACAGAACAAGAGCAAACAAAAGACACAGCAGGCAGCAGCTGCCACAGGAAGCGG
CAGCCAAAATTAC**<u>CCTATAGTGCAAAATGCA</u>**CAAGGGCAAATGGTACATCAGTCCATGTCA
CCTAGGACTTTGAATGCATGGGTGAAGGTAATAGAAGAAAAGGCTTTCAGTCCAGAAGTAA
TACCCATGTTTTCAGCATTATCAGAGGGAGCCACCCCACAAGATTTGAATATGATGCTAAAC
ATAGTGGGGGGACACCAGGCAGCAATGCAGATGCTAAAAGATACCATCAATGAGGAAGCTG
CAGAATGGGATAGGGTACACCCAGTACATGCAGGGCCTATTCCACCAGGCCAGATGAGGGA
ACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAAGAACAAATAGGATGGATG
ACCAGCAATCCACCTATCCCAGTGGGAGAAATCTATAGAAGATGGATAATTCTGGGATTAA
ATAAAATAGTAAGAATGTATAGCCCTGTCAGCATTTTGGACATAAGACAAGGGCCAAAAGA
ACCCTTTAGAGATTATGTAGATCGGTTCTATAAAACTTTGAGAGCTGAACAAGCTACACAGG
AAGTAAAAAATTGGATGACAGAAACCTTGCTGGTCCAAAATGCGAATCCAGACTGTAAGGC
CATTTTAAAATCATTAGGACCAGGGGGCTACATTAGAAGAGATGATGACAGCATGTCAGGG
AGTAGGGGGACCTAGC**CATAAAGCAAGGGTTTTG**GCTGAGGCAATGAGTCAAGCACAACA
GGCCAACATAATGATGCAGAGGGGCAATTTTAGGGGCCAGAGAACAATAAAATGCTTCAAC
TGTGGCAAAGAAGGACATCTAGCCAGAAATTGCAAGGCCCCTAGAAAAAAGGGCTGTTGGA
AATGTGGGAAGGAGGGACACCAAATGAAGGACTGTACTGAGAGACAGGCTAATTTTTTAGG
GAAAATCTGGCCTTCCAGCAAAGGGAGGCCAGGAAATTTTCCTCAGAGCAGACCGGAAACC
AGACCGGAACCAACAG

Figure 3-3. The full length nucleotide sequence of HIV-1_{N20} *gag*. The sequence of HIV-1_{N20} *gag* was amplified from proviral DNA and sequenced by direct sequencing methods. The forward primer sequence used to amplify the *p24* gene is the 18-base sequence in bold/underline, and the reverse primer sequence is the 18-base sequence in bold (no underline).

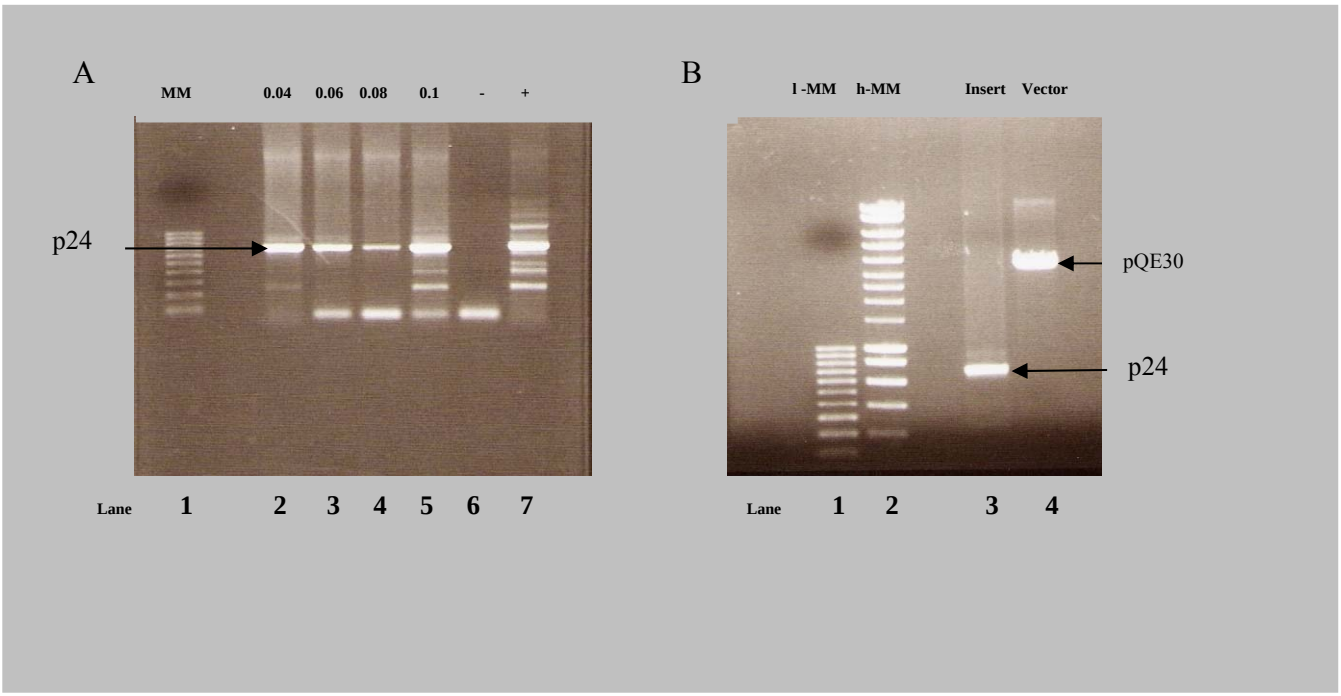


Figure 3-4. Purity of PCR product and insert product of HIV-1_{N20} *p24* gene. In panel A, PCR product amplified from proviral DNA are shown at 0.04 (lane 2), 0.06 (lane 3), 0.08 (lane 4), and 0.1 (lane 5) μg along with negative control (lane 6) and HIV-1-positive control (lane 7) samples at 1 μg. The molecular weight marker (MM) is shown on lane 1. In panel B, the p24 insert (lane 3) and pQE30 vector (lane 4) after restriction enzyme digestion are shown, along with low (lane 1, l-MM) and high (lane 2, h-MM) molecular weight markers. All samples were run on 0.8% tris-acetate EDTA gel for 20 minutes.

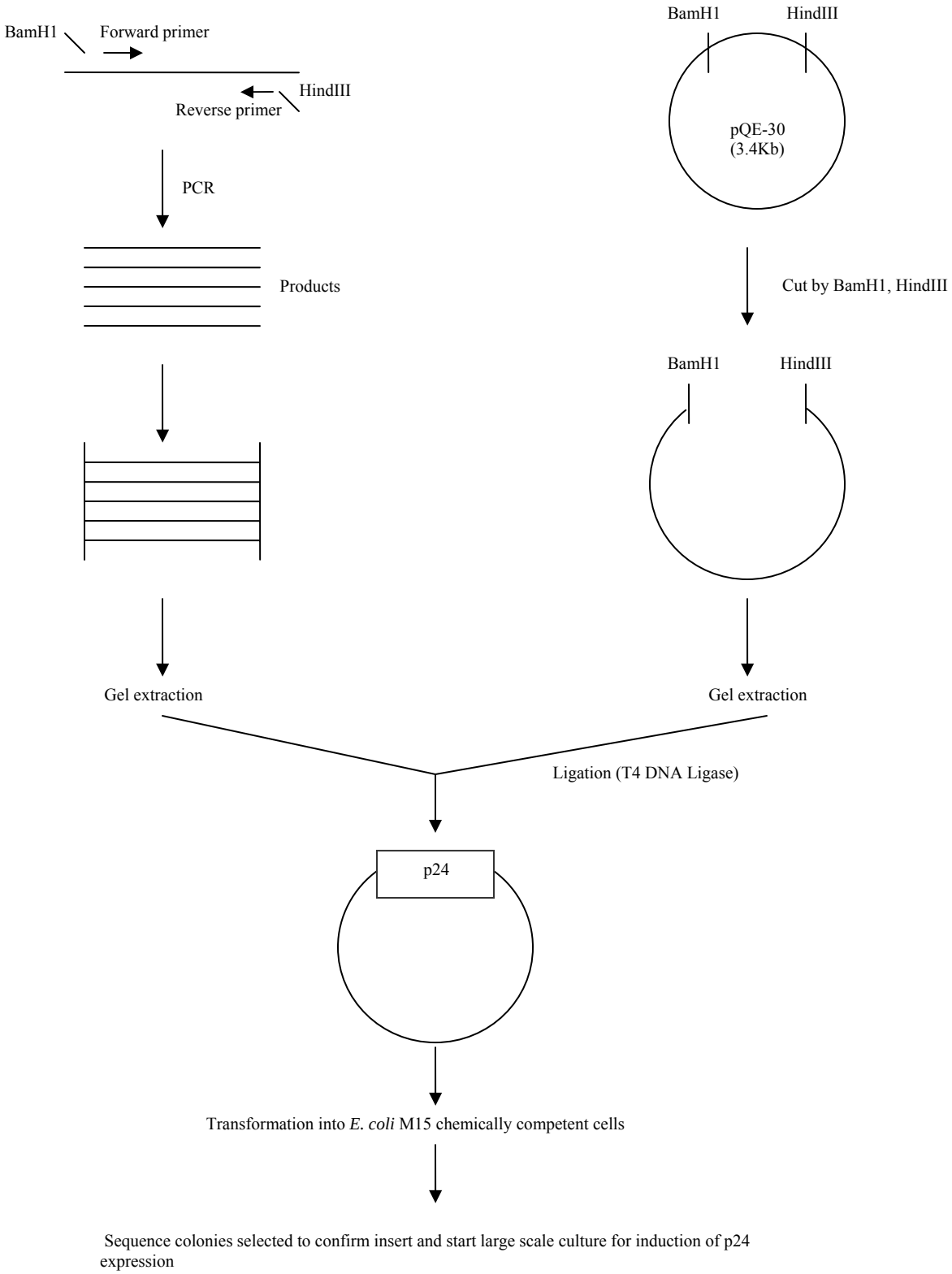


Figure 3-5. Schematic of *p24* gene expression in *E.coli* M15 cells.

BamH1

CGCG/GATCCGCGCCTATAGTGCAAAATGCACAAGGGCAAATGGTACATCAGTCC
ATGTCACCTAGGACTTTGAATGCATGGGTGAAGGTAATAGAAGAAAAGGCTTTCAGTCCA
GAAGTAATACCCATGTTTTCAGCATTATCAGAGGGAGCCACCCCACAAGATTTGAATATG
ATGCTAAACATAGTGGGGGGACACCAGGCAGCAATGCAGATGCTAAAAGATACCATCAAT
GAGGAAGCTGCAGAATGGGATAGGGTACACCCAGTACATGCAGGGCCTATTCCACCAGGC
CAGATGAGGGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAAGAACAA
ATAGGATGGATGACCAGCAATCCACCTATCCCAGTGGGAGAAATCTATAGAAGATGGATA
ATTCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTGTCAGCATTTTGGACATAAGA
CAAGGGCCAAAAGAACCCTTTAGAGATTATGTAGATCGGTTCTATAAAACTTTGAGAGCT
GAACAAGCTACACAGGAAGTAAAAAATTGGATGACAGAAACCTTGCTGGTCCAAAATGCG
AATCCAGACTGTAAGGCCATTTTAAAATCATTAGGACCAGGGGCTACATTAGAAGAGATG
ATGACAGCATGTCAGGGAGTAGGGGGACCTAGCCATAAAGCAAGGGTTTTG**TAGCCC**
A/AGCTTGGG

Lambda Hind III

Figure 3-6. HIV-1_{N20} *p24* sequence with adapter sequences. HIV-1_{N20} *p24* sequence after PCR amplification (sequence without bold) was identical to the *p24* sequence of the *gag* shown in Figure 3-3. The adaptors ligated to the gene create the restriction enzyme sites and are shown in bold on either ends of the gene sequence.

Figure 3-7. Immunoblot and silver-stained gel of the HIV-1_{N20} p24 protein used for Vaccination Study 2. The immunoblots were reacted with either sheep anti-HIV-1 p24 antibodies (panel A) or normal sheep serum (panel B). The immunoblot consisted of HIV-1_{UCD1} p24 (UCD1) at 500 ng (lane 2) and HIV-1_{N20} p24 (N20) at 500 (lane 3), 250 (lane 4), 125 (lane 5), 62.5 (lane 6), 31.25 (lane 7), and 15.62 (lane 8) ng. No non-specific reactivity was observed with normal sheep serum (panel B). The purity of the HIV-1_{N20} p24 preparation was determined by silver-stain analysis of the p24 preparation on 12% SDS-PAGE gel (panel C). The gel consisted of HIV-1_{UCD1} p24 (UCD1) at 500 ng (lane 2) and HIV-1_{N20} p24 (N20) at 500 (lane 3), 250 (lane 4), 125 (lane 5), and 62.5 (lane 6) ng. The gel also contained BSA standards at 25 (lane 7), 12.5 (lane 8), 6.25 (lane 9), and 3.12 (lane 10) ng, which was used to derive the concentration curve. The molecular weight marker (MM) is shown on lane 1 for all panels.

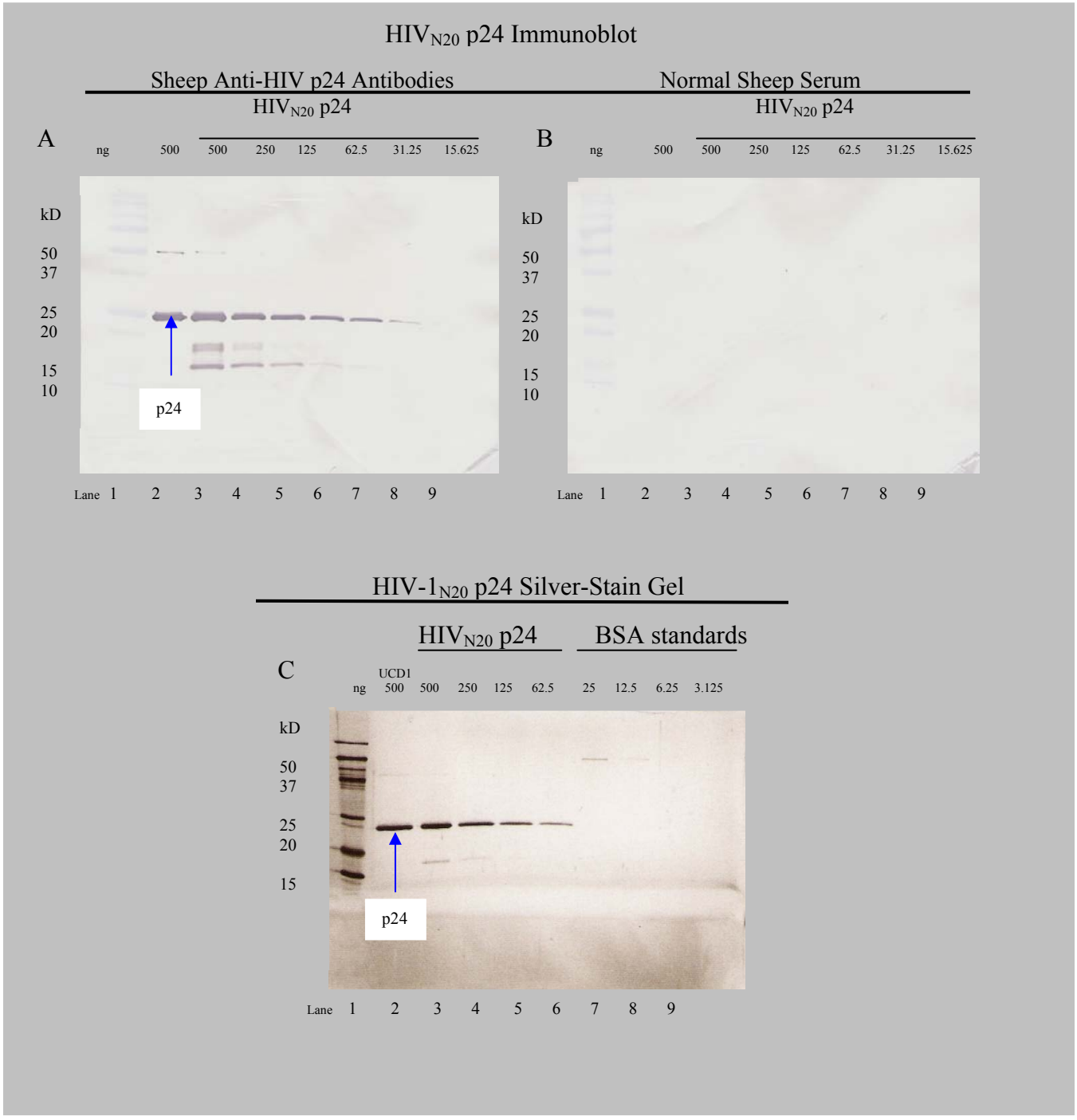

HIV$_{N20}$ p24 Immunoblot
Sheep Anti-HIV p24 Antibodies
Normal Sheep Serum
HIV$_{N20}$ p24
A
B
ng
500
500
250
125
62.5
31.25
15.625
kD
50
37
25
20
15
10
p24
Lane 1 2 3 4 5 6 7 8 9
HIV-1$_{N20}$ p24 Silver-Stain Gel
HIV$_{N20}$ p24
BSA standards
C
UCD1
ng
500
500
250
125
62.5
25
12.5
6.25
3.125
kD
50
37
25
20
15
p24
Lane 1 2 3 4 5 6 7 8 9

Figure 3-8. HIV-1 p24-specific IFNγ responses of vaccinated cats from Vaccination Study 2. IFNγ responses to overlapping peptide pools of HIV-1 (Hp1-Hp18) (A, B, C) were determined by feline IFNγ ELISpot assay. The IFNγ responses of the PBMC from HIV-1_{UCD1} p24 (panel A), HIV-1_{N20} p24 (panel B), FIV_{Pet} p24 (panel C, cats #163, #173, #195), and Fel-O-Vax FIV (panel C, cats #171, #183) vaccinated cats are shown. The virus-specific positive controls included IFNγ responses to recombinant HIV-1_{UCD1} p24 (UCD1 p24), HIV-1_{N20} p24 (N20 p24), and FIV_{Pet} p24 (Pet p24) proteins, and inactivated dual-subtype FIV whole-viruses (IWV). The PBMC from all vaccinated cats had moderate-to-high levels of responses to T-cell mitogen stimulation with ConA (data not shown). ELISpot assays to both HIV-1 and FIV peptides were performed at the same time. As a result, the positive control values for set 1 and set 2 of each vaccination group are identical. No IFNγ responses to LPS and FeT-J cell lysate were detected in the PBMC from vaccinated cats, with the exception of those from Fel-O-Vax FIV-vaccinated cats, which responded to cell lysate. The IWV-stimulation results of PBMC from Fel-O-Vax FIV-vaccinated cats are shown as SFU values after subtracting out the SFU values for FeT-J cell lysate stimulation. SFU values (bars) above or at the dashed red line at 50 SFU (stringent threshold) were considered significant.

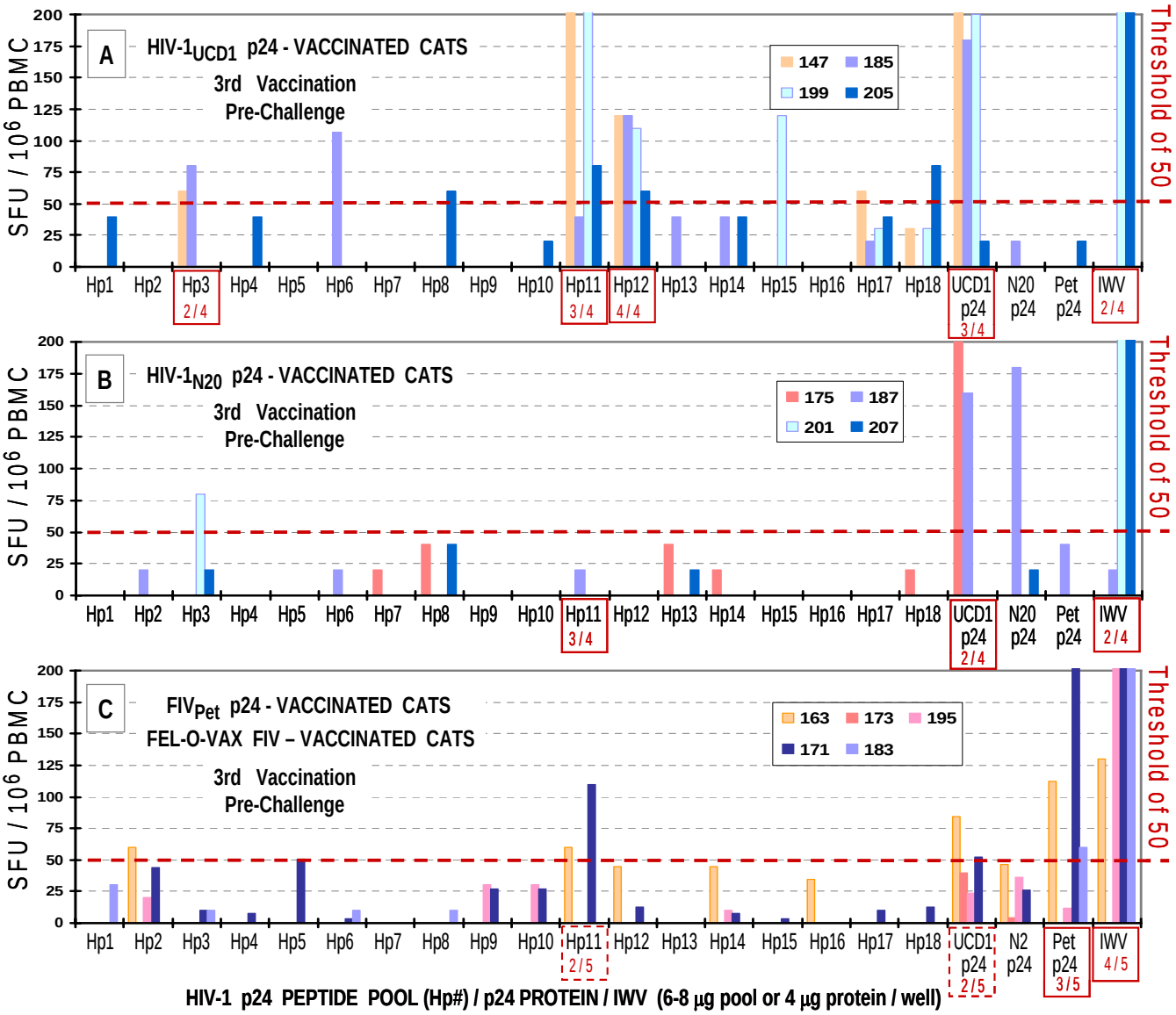
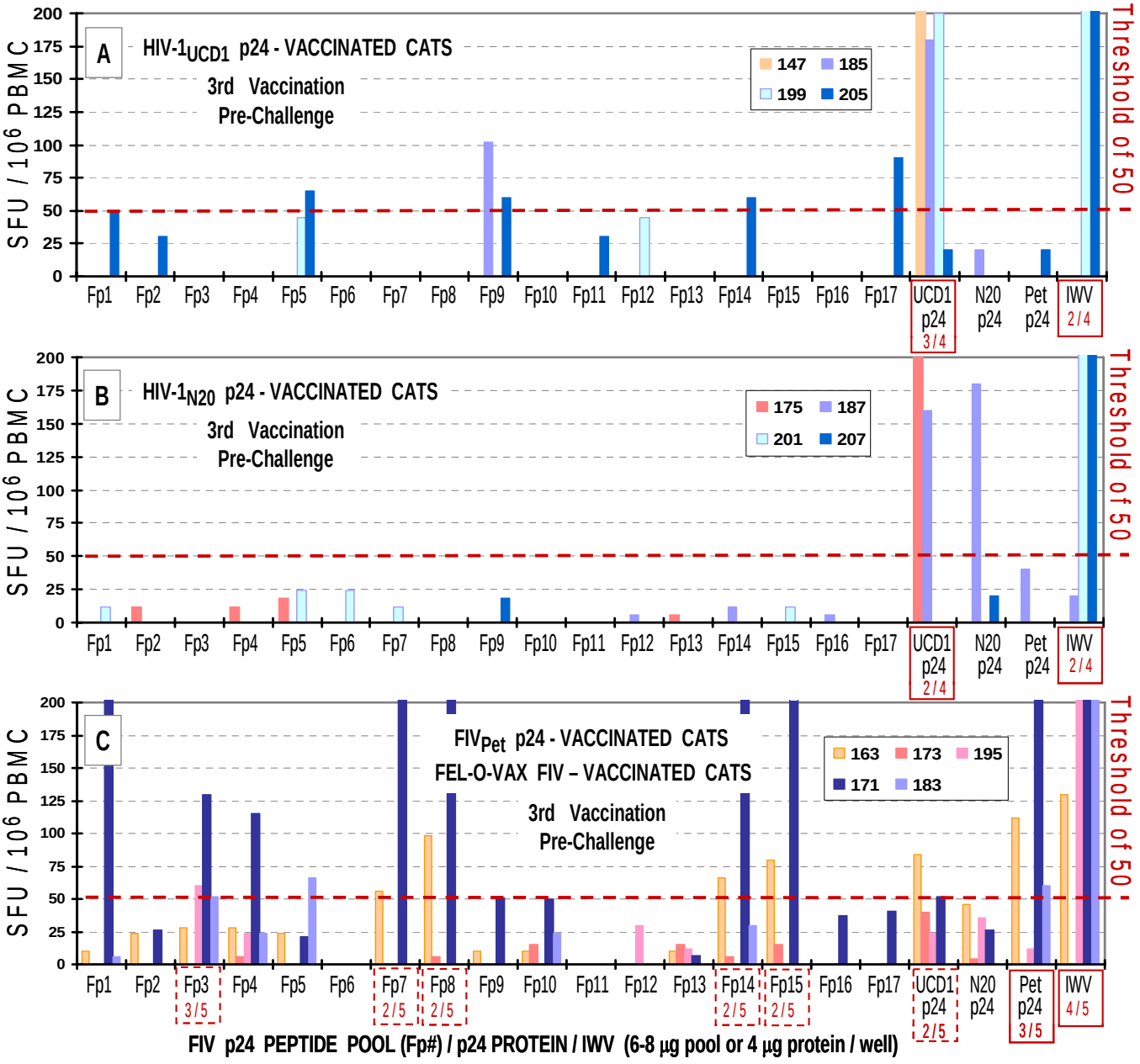

A
HIV-1UCD1 p24 - VACCINATED CATS
3rd Vaccination
Pre-Challenge
147 185 199 205
B
HIV-1N20 p24 - VACCINATED CATS
3rd Vaccination
Pre-Challenge
175 187 201 207
C
FIVPet p24 - VACCINATED CATS
FEL-O-VAX FIV – VACCINATED CATS
3rd Vaccination
Pre-Challenge
163 173 195 171 183
SFU / 10^6 PBMC
Threshold of 50
Hp1 Hp2 Hp3 2/4 Hp4 Hp5 Hp6 Hp7 Hp8 Hp9 Hp10 Hp11 3/4 Hp12 4/4 Hp13 Hp14 Hp15 Hp16 Hp17 Hp18 UCD1 p24 3/4 N20 p24 Pet p24 IWV 2/4
Hp1 Hp2 Hp3 Hp4 Hp5 Hp6 Hp7 Hp8 Hp9 Hp10 Hp11 3/4 Hp12 Hp13 Hp14 Hp15 Hp16 Hp17 Hp18 UCD1 p24 2/4 N20 p24 Pet p24 IWV 2/4
Hp1 Hp2 Hp3 Hp4 Hp5 Hp6 Hp7 Hp8 Hp9 Hp10 Hp11 2/5 Hp12 Hp13 Hp14 Hp15 Hp16 Hp17 Hp18 UCD1 p24 2/5 N2 p24 Pet p24 3/5 IWV 4/5
HIV-1 p24 PEPTIDE POOL (Hp#) / p24 PROTEIN / IWV (6-8 µg pool or 4 µg protein / well)

Figure 3-9. FIV p24-specific IFNγ responses of vaccinated cats from Vaccination Study 2. IFNγ responses to overlapping peptide pools of FIV p24 (Fp1-Fp17) (A, B, C) were determined by feline IFNγ ELISpot assay. The IFNγ responses of the PBMC from HIV-1_{UCD1} p24 (panel A), HIV-1_{N20} p24 (panel B), FIV_{Pet} p24 (panel C, cats #163, #173, #195), and Fel-O-Vax FIV (panel C, cats #171, #183) vaccinated cats are shown. The virus-specific positive controls included IFNγ responses to recombinant HIV-1_{UCD1} p24 (UCD1 p24), HIV-1_{N20} p24 (N20 p24), and FIV_{Pet} p24 (Pet p24) proteins, and inactivated dual-subtype FIV whole-viruses (IWV). The PBMC from all vaccinated cats had moderate-to-high levels of responses to T-cell mitogen stimulation with ConA (data not shown). ELISpot assays to both HIV-1 and FIV peptides were performed at the same time. As a result, the positive control values for set 1 and set 2 of each vaccination group are identical. No IFNγ responses to LPS and FeT-J cell lysate were detected in the PBMC from vaccinated cats, with the exception of those from Fel-O-Vax FIV-vaccinated cats, which responded to cell lysate. The IWV-stimulation results of PBMC from Fel-O-Vax FIV-vaccinated cats are shown as SFU values after subtracting out the SFU values for FeT-J cell lysate stimulation. SFU values (bars) above or at the dashed red line at 50 SFU (stringent threshold) were considered significant.


A
HIV-1UCD1 p24 - VACCINATED CATS
3rd Vaccination
Pre-Challenge
147 185 199 205
SFU / 10^6 PBMC
Threshold of 50
Fp1 Fp2 Fp3 Fp4 Fp5 Fp6 Fp7 Fp8 Fp9 Fp10 Fp11 Fp12 Fp13 Fp14 Fp15 Fp16 Fp17 UCD1 p24 3/4 N20 p24 Pet p24 IWV 2/4
B
HIV-1N20 p24 - VACCINATED CATS
3rd Vaccination
Pre-Challenge
175 187 201 207
SFU / 10^6 PBMC
Threshold of 50
Fp1 Fp2 Fp3 Fp4 Fp5 Fp6 Fp7 Fp8 Fp9 Fp10 Fp11 Fp12 Fp13 Fp14 Fp15 Fp16 Fp17 UCD1 p24 2/4 N20 p24 Pet p24 IWV 2/4
C
FIVPet p24 - VACCINATED CATS
FEL-O-VAX FIV – VACCINATED CATS
3rd Vaccination
Pre-Challenge
163 173 195 171 183
SFU / 10^6 PBMC
Threshold of 50
Fp1 Fp2 Fp3 3/5 Fp4 Fp5 Fp6 Fp7 2/5 Fp8 2/5 Fp9 Fp10 Fp11 Fp12 Fp13 Fp14 2/5 Fp15 2/5 Fp16 Fp17 UCD1 p24 2/5 N20 p24 Pet p24 3/5 IWV 4/5
FIV p24 PEPTIDE POOL (Fp#) / p24 PROTEIN / IWV (6-8 µg pool or 4 µg protein / well)

CHAPTER 4
FINAL DISCUSSION

The long-term goal of our studies is the development of a highly efficacious HIV-1 vaccine. In our initiative, our laboratory seeks to develop a blueprint to this objective by understanding the mechanism of our HIV-1 p24 vaccine efficacy. Our dual-subtype model using inactivated whole-virus, though highly successful, presents several safety concerns (i.e., incomplete inactivation) which limit its use in HIV-1 vaccines for humans. Understanding the mechanism of protection of our HIV-1 p24 vaccine provides a novel opportunity to establish the minimum epitopic requirements necessary for a protective vaccine. This is important since several safety issues prevent the use of a full virus construct.

HIV-1 p24 vaccine was able to provide protection against FIV, while FIV p24 vaccine had minimal efficacy. This result seems counter-intuitive, since there is considerably more sequence identity and homology between FIV p24 and FIV challenge virus than HIV-1 p24 and FIV challenge virus. This result indicates that current vaccine dogma of including conserved epitopes may not be sufficient for the successful design of an HIV-1 vaccine. Identifying and omitting the immunodominant non-protective epitopes, which may detract the establishment of an effective antiviral immune response, also may be necessary.

Core protein may be only a part of the overall vaccine equation. Establishment of an efficient HIV-1 vaccine may require the utilization of multiple virus proteins in a subunit or vector vaccine. Currently, our laboratory is engaged in examination of the cellular immune response of cats to HIV-1 p24 and other conserved HIV-1 p24 proteins (RT and MA). The aim is to identify the epitopes that are associated with cross-protection and are recognized by both cats and humans. The results show that both cats and humans recognize the KK10 epitope of HIV-1. If the feline immune response is similar to that of humans then HIV-1/FIV-cat model provides us

with a means of selecting desirable epitopes for inclusion in an HIV-1 vaccine. It is not yet clear if this epitope is protective. Though our results are promising, more studies will have to be performed in order to identify the specific protective epitopes.

The findings from current studies demonstrated that protection of the HIV-1/FIV vaccine is based on cellular immunity. The exact immune cells involved in this protection are still unknown. The cells capable of IFNγ responses include not only CTL but T-helper (TH1) and NK cells [64]. It is necessary to perform studies with enriched populations of CTL, TH, and NK cells to determine which cells are primarily responsible for the vaccine efficacy and which peptides are recognized. This could also provide us with the means to determine if the epitopes involved in FIV protection and improved HIV-1 prognosis are the same.

The current studies illustrate that it is possible to develop an efficient FIV vaccine using HIV-1 p24. These vaccines were consistently able to outperform vaccines based on homologous virus p24. This presents the possibility of developing a second-generation FIV vaccine for veterinary medicine, which has broader efficacy than our commercial FIV vaccine and addresses the problem of conflict with currently available diagnostics faced by our commercial vaccine (Fel-O-Vax FIV vaccine).

The findings of the passive-transfer study (Chapter 1) indicated that the antibody immunity to HIV-1 based FIV vaccines was insufficient to provide protection against FIV challenge. This implies that the protection observed with our HIV-1 p24 based vaccine was the result of the host cellular response. Further examination of this response is required to fully understand the mechanism of the vaccine. These findings of cross-species protection of FIV provide an opportunity to utilize the cat model to determine if the immunodominant hypothesis is

valid and if so the mechanism of utilizing this selection criteria for the formulation of an HIV-1 vaccine.

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BIOGRAPHICAL SKETCH

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