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Fowlpox virus vaccines for HIV and SHIV clinical and pre-clinical trials

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Abstract

DNA prime and recombinant fowlpox virus (rFPV) boost vaccines were designed to express multiple HIV or SIV antigens for use in human clinical trials and in pre-clinical trials in macaques. Three sets of vaccines with matching HIV or SIV antigen sets, modified for vaccine safety considerations, were constructed and shown to express the relevant proteins. The rFPV vaccines with inserts at up to three sites, were stable on passage in chick cell culture, including during GMP manufacture of vaccines for human Phase I clinical trials. Cellular and humoral immunogenicity in mice was demonstrated using a DNA prime/rFPV boost and vaccinia virus challenge model. These data establish a preliminary safety and efficacy profile for these multigenic vaccines suggesting they are suitable for advanced development as candidate HIV vaccines.

Keywords: HIV vaccine; Recombinant fowlpox virus; DNA; Prime/boost

1. Introduction

In the search for effective vaccines against HIV-1 a broadly reactive neutralising antibody response has proved elusive to date, while some success in generating HIV-specific T-cell responses suggests these may provide an option for control of the infection. Cytotoxic T lymphocytes (CTL) specific for HIV-1 antigens, which correlate with the control of acute viremia, have been detected in primary HIV-1 infection, in the absence of neutralising antibody activity [1]. Depletion of CD8 T cells in SIV infected macaques results in an inability to control viremia [2], induction of SHIV-specific T cells correlates with protective immunity [3] and HIV-specific helper T cells may also play a role in protective immunity [4].

A number of strategies for stimulating T-cell responses to a range of antigens, including some from HIV, have involved sequential priming of the immune response with one vaccine and boosting with the same or similar antigens delivered with another vector [5–11], leading to higher responses than with either vaccine alone [5,11]. Many of these studies have involved priming with a DNA plasmid and boosting with recombinant poxviruses, including fowlpox virus (rFPV) [5,6].

Further enhancement of HIV vaccine immunogenicity using molecular approaches to providing immunomodulatory adjuvants is currently being explored by many groups. Coexpression of cytokines, either from a viral vector [12–14], or from DNA plasmids [3], has been shown to enhance cell-mediated immune responses in mice [12,13] and in macaques [3,14].

Initial studies using DNA priming and rFPV boosting have shown that HIV specific T-cell responses can be stimulated in mice and in macaques [5] using subtype B HIV-1 Gag and Pol. In order to progress these studies to human clinical trials a number of modifications and refinements of the DNA and rFPV vaccines need to be undertaken. Three sets of vaccines, DNA and rFPV, with expressed safety-modified HIV or SIV sequences matched as closely as possible, have been constructed: (1) based on a subtype B HIV-1 for clinical trial

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in Sydney; (2) based on subtype AE HIV-1 for clinical trials in South East Asia, where this subtype is the predominant circulating strain; and (3) SHIV constructs for preclinical macaque trials, in particular a rigorous assessment of protection from SIV or SHIV challenge. Modifications to the HIV sequences to address safety concerns and regulatory issues have been included in the constructs, with the detailed results of the safety testing to be reported elsewhere [Purcell et al., manuscript in preparation]. Here, we report the construction of rFPV vaccines, designed to boost immune responses after DNA priming, and their characterisation including genome stability of the rFPV, expression of antigens and murine immunogenicity.

2. Materials and methods

2.1. Modifications to HIV sequences for DNA vaccines

Source materials for HIV and SIV sequences were full-length molecular clones as indicated in Table 1. Specific modifications to HIV sequences were designed to remove the RNA-binding zinc finger motifs from the nucleocapsid region of Gag, and to abolish reverse transcriptase (RT) activity, RNase H activity and integrase activity from Pol and proviral long terminal repeats (LTRs) as detailed elsewhere [Purcell et al., manuscript in preparation]. Modified HIV sequences for subtype B and subtype AE were inserted in the DNA expression vector pHIS64 (Coley Pharmaceuticals Inc.) which uses the CMV immediate early promoter and the bovine growth hormone polyadenylation signal, and includes an artificial intron, 16 copies of a primate CpG motif (24-mer) and a kanamycin resistance gene for plasmid selection. The subtype B DNA vaccine (pHIS-HIV-B) expressed modified Gag and GagPol by translational frameshifting, a truncated version of Env (243 amino acids) resulting from adding an in-frame stop codon to replace 581 bp including the V3 and CD4 binding domains, and truncated Nef (36 amino acids), full sequences for Vpu, Tat and Rev, and deletions that eliminated the coding regions for Vpr, Vif and the LTRs. The subtype AE DNA vaccine (pHIS-HIV-AE) contained the corresponding sequence modifications with the exception that Vpu was not expressed, due to a frameshift mutation, and the AE expressed a truncated Env of 675 amino acids resulting from the preservation of the reading frame after deleting 581 bp including the V3 and CD4 binding domains.

2.2. Recombinant FPV construction

Further modifications of HIV and SIV sequences for optimal expression in recombinant fowlpox viruses were as outlined in Table 1. Plasmid DNA (pHIS-HIV-B or -AE) was used to generate the appropriate fragments by PCR with the addition of a fowlpox virus early/late promoter ($P_{E/L}$) in frame with the initiation codon and a poxvirus early transcription terminator (T_5NT) at the 3′-end of the continuous coding sequence. PCR products were cloned in pGEM-T Easy (Promega) and the sequence, with modifications included in the DNA vaccines, was confirmed prior to cloning in rFPV insertion plasmids, pKG10a, pCH34, pAF09 or pAFtd as described elsewhere [15]. Within the AE *env* sequence T_5NT (537–543) was converted to CTTGTAT to avoid premature transcription termination in the poxvirus

Table 1
Modifications to HIV and SIV genes for FPV expression

HIV or SIV subtype source of genetic material	FPV insert	Modifications to sequence for vaccine safety	Modifications for FPV expression
HIV-1 subtype B pNL(AD8)	B *gagpol*	Unmodified	FPV promoter $P_{E/L}$ & terminator T_5NT
	B *gagpol* (mut)	Deleted: *gag* zinc finger motifs; *pol* RT active sites; RNase H active site; integrase function.	FPV promoter $P_{E/L}$ & terminator T_5NT
	B *env* (mut)	Deleted: CD4 binding domain	Fusion of amino acids 1–270 with 751–855 (375 amino acids); FPV promoter $P_{E/L}$ & terminator T_5NT
	B *tat* & *rev*	Unmodified	Spliced *tat* exons to single ORF (86 amino acids); spliced *rev* exons to single ORF (116 amino acids); mutated 1st 180 bp of *rev*; fused *tat* to *rev* via six His residues (208 amino acid ORF); FPV promoter $P_{E/L}$ & terminator T_5NT
HIV-1 subtype AE p93TH253	AE *gagpol* (mut)	Deleted: *gag* zinc finger motifs; *pol* RT active sites; RNase H active site; integrase function.	FPV promoter $P_{E/L}$ & terminator T_5NT
	AE *env* (mut)	Deleted: CD4 binding domain	Mutation to remove internal T_5NT; FPV promoter $P_{E/L}$ & terminator T_5NT
	AE *tat* & *rev*	Unmodified	Spliced *tat* exons to single ORF (102 amino acids); spliced *rev* exons to single ORF (124 amino acids); mutated 1st 180 bp of *rev*; fused *tat* to *rev* via five His residues (231 amino acid ORF); FPV promoter $P_{E/L}$ & terminator T_5NT
SIV_{mac239}	SIV *gagpol*	Unmodified	FPV promoter $P_{E/L}$ & terminator T_5NT

system. Several PCR derived point mutations were identified and non-synonymous changes were corrected.

While the HIV DNA vaccines express Tat and Rev from alternatively spliced mRNAs that share considerable sequence, FPV expression of both Tat and Rev required fusing these exons and eliminating extensive sequence repeats. The exons for AE Tat were fused to a single ORF (102 amino acids) and inserted in pAFtd as a promoter-gene-terminator cassette. Fusion of the exons for Rev was carried out in a similar manner, however, all possible conservative changes were introduced in the first 180 bp of Rev, to avoid duplication of sequences from Tat and to generate an ORF of 124 amino acids. Finally, the stop codon of Tat was removed and Tat and Rev sequences were fused via five histidine residues. The fusion construct (AEtatHrev) with 231 amino acids of continuous coding sequence, and with promoter and terminator sequences added, was inserted in pAF09. Similarly a fusion of Tat and Rev from HIV subtype B was constructed where 86 amino acids of Tat sequence was fused via six His residues to 116 amino acids of Rev. The resultant NLtatHrev (208 amino acids) with promoter and terminator, was inserted in rFPV plasmid pAFtd.

Recombinant FPVs were constructed using transient dominant or dominant selection protocols for the insertion of genetic material at up to three sites in the viral genome of FPV-M3 [16] as detailed elsewhere [15]. pKG10a was used for the insertion of the modified HIV B or AE *gagpol* sequences at the F6, 7, 9 locus in FPV-M3, while pCH34 was used to insert the modified HIV AE *env* sequence at the REV site. Where cytokine genes (human or murine, IFNγ or IL12) were included the dominant selection vector pAF09 was used for insertion between the FPV thymidine kinase (TK) and the adjacent uncharacterised gene (ORF X), resulting in the retention of the marker genes for *Eco*gpt and β-galactosidase.

2.3. Recombinant vaccinia virus construction

Vaccinia virus (VV) plasmid pUC-J [17] was modified by deletion of 20 bp and insertion of a multiple cloning site at the *Eco*RI site within the VV TK gene. The resultant plasmid, pJmcs, contained unique sites for *Cla*I, *Sal*I, *Hin*cII, *Eco*RI, *Nco*I, *Sty*I, *Not*I, *Ava*I, *Xho*I, *Bss*HI and *Bam*HI suitable for the insertion of promoter-gene cassettes and the construction of VV transfer plasmids. Promoter-gene-terminator cassettes for the modified HIV subtype AE genes, identical to those used for rFPV constructs and using the FPV early/late promoter $P_{E/L}$, were inserted between the *Sal*I and *Xho*I sites of pJmcs to generate pJmcs-AEgagpol, pJmcs-AEenv and pJmcs-AEtatHrev. These plasmids were used to transfect human 143B (TK^-) cells [18] previously infected with VV-WR-L929 [17] and TK negative recombinants were selected in the presence of BUdR as has been described [17]. Recombinant viruses were plaque purified in the absence of selective medium and using a SYBR Green sequence detection assay as described in Boyle et al. [15]. A recombinant virus, VVARVTK, expressing an unmodified version of HIV-1 subtype B SF2 GagPol, inserted within the TK of VV-WR-L929 and under the control of the VV early/late promoter $P_{7.5}$, has been described [5].

2.4. Genome characterisation and sequencing

The genomic configuration of recombinant viruses (rFPV and rVV) and confirmation of plaque purification, was determined by PCR using sequences within the poxvirus flanking regions for each insertion site [15]. Primer sequences for rFPV insertions have been reported [15], while for rVV insertions primers Jseq1 (CCATCGAGTGCGGCTACTAT) and Jseq2 (ACGGCGGACATATTCAGTTG), which generated a PCR fragment of 328 bp on non-recombinant virus, were used. FPV recombinants intended for human clinical trials (rFPV-HIV-B and rFPV-HIV-AE) were fully sequenced across all inserts and their flanking regions, both before and after the manufacturing process.

2.5. Western blotting

Confluent monolayers of chick embryo skin (CES) cells or CV-1 (African green monkey kidney) cells were infected with rFPV at a multiplicity of infection (m.o.i.) of 5 pfu per cell. Cell pellets and supernatants were harvested at 48–96 h post-infection. Cell lysates were prepared by scraping infected cell monolayers into 10 ml of phosphate buffered saline (PBS), centrifugation at $2500 \times g$ for 10 min at 4 °C and resuspension of the pelleted material in 300 μl of PBS. Supernatants from infected cell cultures were filtered (0.2 μm) to remove poxvirus particles, and concentrated by centrifugation at 25,000 rpm (80–100,000 × *g*) for 3.5 h at 4° through a cushion of 20% sucrose/Tris–EDTA pH 8 using a Beckman SW28 rotor, prior to analysis by denaturing gel electrophoresis on NuPAGE® Novex Bis-Tris gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with appropriate antiserum. Chemiluminescence with peroxidase-labelled secondary antibodies or protein A/G was used for detection. Molecular weight markers, MagicMark™ Western Standard and SeeBlue® Plus2 Pre-Stained Standard (Invitrogen), were included on all gels.

2.6. Tat expression

CEM-GFP reporter cells for HIV Tat (AIDS Reference Reagent Program #3655, a CD4+ T cell line stably transfected with LTR-GFP [19]) were infected with rFPV at a m.o.i. of 10 pfu per cell in medium containing G418. At various times post-infection cells were tested for green fluorescent protein (GFP) expression using a FACSCalibur with CELL-Quest software for analysis (Becton Dickinson, Mountain View, CA). Data were expressed as relative fluorescence (percent positive cells × median channel fluorescence).

2.7. p24 ELISA and antibody ELISAs

Quantitation of HIV p24 antigen was carried out using an Innotest™ HIV Antigen mAB kit (Innogenetics N.V., Ghent, Belgium) and the p24 standard provided with the kit. Serum antibody to p24 was determined by ELISA using HIV-1 subtype B recombinant p24 (Intracel Corp, Carisbad, CA) and titres were expressed as the highest dilution providing an OD > 0.3 (negative serum + 2 standard deviations). Serum antibody to Env was tested by ELISA using a purified recombinant HIV-1 subtype AE Env protein modified to mimic the vaccine construct (i.e. with the V3 region deleted) and lacking the transmembrane domain. Positive control serum was provided by serum from a mouse vaccinated with HIV-1 subtype B Env gp140.

2.8. Cytokine ELISAs

Supernatants from rFPV infected cells were tested for the presence of individual cytokines (human and murine, IFN-γ and IL-12) using R&D Systems Quantikine® Immunoassay kits and the standards provided. Supernatants from CES cells infected with FPV-M3 were used as negative controls in each case.

2.9. Mouse inoculations and VV challenge

Groups of six or seven female BALB/c mice, 6–8-weeks old, were inoculated intramuscularly (IM) with 100 μg of DNA vaccine at weeks 0 and 4 and boosted at week 8 with 5×10^6 pfu of rFPV vaccine by the intraperitoneal (IP) route. Vaccinia virus challenge at week 9 was IP with 10^7 pfu of virus. Mice were sacrificed at day 4 post-challenge when ovaries were removed for VV titration and blood was collected for serum antibody testing. VV titration was by plaque assay on human 143B cells as described previously [17]. *p*-Values were obtained using a two-tailed two sample equal variance Student's *t*-test.

3. Results

3.1. Construction of FPV recombinants and their genomic stability

FPV recombinants with combinations of HIV, SIV and cytokine genes inserted at three locations in the viral genome have been constructed and are shown in Table 2. In each case the genomic configuration, with respect to size and location of inserted DNA, was confirmed by PCR using primers to FPV flanking sequences for each insertion site. DNA fragments derived by PCR using FPV-117 (rFPV-HIV-AE vaccine) are shown in Fig. 1 compared with the control or parental virus (FPV-M3). In each case the PCR product from FPV-117 (lanes marked 1) is larger than the product from FPV with no insertion at that site (lanes marked M3) demonstrating the presence of additional material of the predicted size at each location. No evidence of non-recombinant FPV (as would be the case in the event of incomplete plaque purification) or breakdown products, where in either case smaller fragments would be more readily amplified, was detected. In addition to providing confirmation of the genomic configuration, PCR products from subsequent passages of FPV-117 in CES cell culture were compared (lanes 2 and 3 for each insertion site). The data in Fig. 1 show that genomic stability was maintained when the plaque-purified rFPV was amplified to provide stocks. The sequence of the inserted DNA and the FPV flanking sequences (>180 bp each side of the insertion site) was confirmed for critical constructs including FPV-086 and FPV-117 (rFPV-HIV-B and -AE vaccines) intended for use in human clinical trials. No mutations or changes were detected when the sequence was compared with that

Table 2
Fowlpox virus recombinants expressing HIV and SIV genes

Virus number	Descriptive name	Genes expressed from FPV insertion sites[a]		
		F6, 7, 9 site (pKG10a)	REV site (pCH34)	TK ORF X site (pAF09/pAFtd)
FPV-059	rFPV-HIV-B wild type	B *gagpol*		
FPV-086	rFPV-HIV-B vaccine	B *gagpol* (mut)		
FPV-087	rFPV-HIV-B + huIFNγ	B *gagpol* (mut)		huIFN-γ (*Eco*gpt–β-gal)
FPV-088	rFPV-HIV-B + huIFNγ	B *gagpol* (mut)		huIL-12 (*Eco*gpt–β-gal)
FPV-119	rFPV-HIV-B Env + Tat–Rev	B *env* (mut)		B *tat–rev* fusion
FPV-117	rFPV-HIV-AE vaccine	AE *gagpol* (mut)	AE *env* (mut)	AE *tat–rev* fusion (*Eco*gpt–β-gal)
FPV-100	rFPV-HIV-AE + huIFN-γ	AE *gagpol* (mut)	AE *env* (mut)	huIFN-γ (*Eco*gpt–β-gal)
FPV-106	rFPV-HIV-AE + muIFN-γ	AE *gagpol* (mut)	AE *env* (mut)	muIFN-γ (*Eco*gpt–β-gal)
FPV-107	rFPV-HIV-AE + muIL-12	AE *gagpol* (mut)	AE *env* (mut)	muIL-12 (*Eco*gpt–β-gal)
FPV-090	rFPV-HIV-AE Env		AE *env* (mut)	
FPV-096	rFPV-HIV-AE Tat			AE *tat*
FPV-123	rFPV-S/HIV vaccine	SIV *gagpol*	AE *env* (mut)	AE *tat–rev* fusion (*Eco*gpt–β-gal)
FPV-089	rFPV-SIV GagPol	SIV *gagpol*		
FPV-094	rFPV-SIV GagPol + huIFN-γ	SIV *gagpol*		huIFN-γ (*Eco*gpt–β-gal)

[a] Insertion sites and FPV plasmids described in Boyle et al. [15].

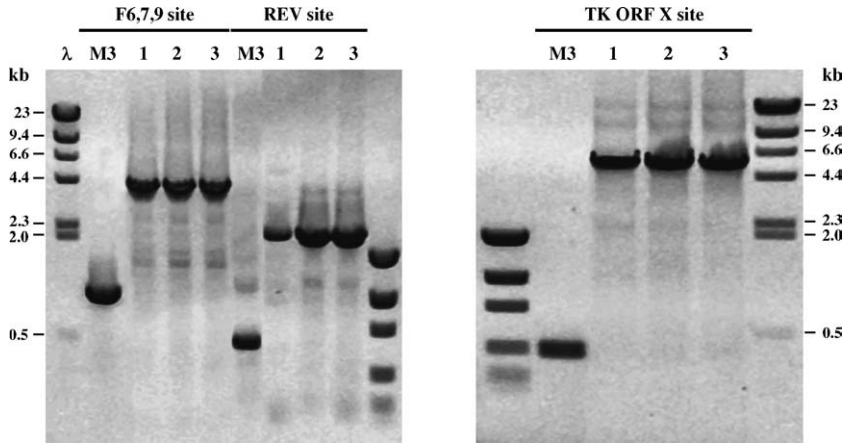

Fig. 1. Confirmation of recombinant FPV genomic structure and stability. PCR fragments showing insertions at three sites in FPV genome after serial passage in CES cell culture. Agarose gel electrophoresis of PCR products from FPV-117 compared with parental FPV-M3 (lanes M3) using insertion site-specific primer pairs as described previously [15]. Lanes marked 1, 2, and 3 indicate FPV-117 DNA template prepared after one, two, or three rounds of viral amplification in CES cell culture, respectively.

of the plasmid used to construct the recombinant virus (data not shown). Further passages for large-scale manufacture of FPV-086 (rFPV-HIV-B vaccine) and FPV-117 (rFPV-HIV-AE vaccine) were similarly tested. The genome configuration and sequence of inserted material was unchanged after a total of five passages for FPV-086 and after four passages for FPV-117 (data not shown).

3.2. Expression of HIV and SIV antigens from FPV recombinants

Proteins from cells infected with rFPVs expressing HIV and SIV genes were analysed by Western blot with appropriate HIV-specific antisera. Fig. 2 shows representative gels derived from rFPV-infected avian (CES) or mammalian (CV-1) cell cultures. The products of both *gag* and *env* gene insertions were detected using patient antiserum, while a monoclonal antibody to p24 was used to detect differences in the level of processing of *gag* and *gagpol* gene products expressed. In Fig. 2A varying degrees of processing of Gag p55 to the mature p24, can be seen in material collected from the supernatants of rFPV-infected cell cultures. A greater proportion of processed product was derived from unmodifed GagPol constructs (FPV-059 and FPV-123) suggesting that mutation of the NC and RT regions of GagPol and removal of the IN region of Pol diminished the proteolytic activity of the PR region that was not directly altered by any of the introduced mutations. Similar profiles resulted from supernatants from rFPV infection of mammalian cells when compared with those from avian cells (Fig. 2A). In comparison, total cell lysates from infected cells showed similar patterns of Gag proteins but with additional low molecular weight bands in avian cell extracts (data not shown). Fig. 2B shows direct comparisons of products from supernatants of infected CES and CV-1 cells infected with FPV-059 (unmodified B Gag) and FPV-086 (mutated B Gag) at 48 and 96 h post-infection. The data suggest that more efficient processing occured in avian cells than in mammalian cells and that the mutations introduced into *gag* and *pol* sequences have reduced both the level of expression and the degree of proteolytic processing, most notably in infected CV-1 cells.

For quantitative analysis of Gag expression, cell lysates and concentrated supernatants from rFPV-infected CES and CV-1 cells were tested in an ELISA kit specific for p24 from HIV-1 subtype B (Table 3). Results are expressed in pg of p24 per 10^6 cells, irrespective of the differences in the sizes and numbers of cells used in the infection (15×10^6 of the smaller CES cells versus 6×10^6 of the larger CV-1 cells). Cell lysates from CV-1 cell infections with FPV-059, FPV-117 and FPV-123 showed approximately two-fold higher levels of p24, than was detected from CES cell infections. In contrast, FPV-086 infections of CES cells showed higher levels of p24 than was observed with CV-1 cells. In infections with any of the four constructs tested, the percentage of p24 released into the culture supernatant was greater from CES cells than from CV-1 cells. Comparisons between the two forms of subtype B Gag, within each cell type showed 4–5-fold reductions in the percentage of p24 detected in the culture supernatants in both cell types for the mutated construct (FPV-086) compared with FPV-059 (Table 3). The monoclonal antibody used for quantitation was specific for p24 of HIV-1 subtype B and its reactivity against Gag products derived from HIV-1 subtype AE or from SIV was not known or tested. This might account for the lower levels of expression from FPV-117 and FPV-123 in both cell types.

Products of the modified *env* genes (HIV-1 B and AE) were detected by Western blotting with antiserum directed against recombinant subtype B gp120. Fig. 2C shows supernatants from infected avian and mammalian cells where the AE *env*, modified to produce a 675 amino acid protein, was expressed from two FPV recombinants. The pattern of bands detected suggests that more efficient processing occurred in the avian CES cells. In lysates from CES cells infected with FPV-119, where a more truncated version of the subtype B Env (375

amino acids) is expressed, a single band was detected (data not shown).

Protein products from cell lysates infected with FPV recombinants containing Tat–Rev fusion constructs are shown in Fig. 2D. Antiserum to the histidine linker was used to detect products from both AE and B Tat–Rev fusions with a stronger signal arising from the six His residues in the B construct compared with five His residues in AEtatHrev. Antiserum to Tat was specific for subtype B and did not detect the AE Tat products on Western blot (Fig. 2E). Since the AE Tat expressed from FPV-096, also lacked the His linker, detection by Western blot was precluded. On the other hand, anti-Rev serum was cross-reactive for both subtypes (Fig. 2F).

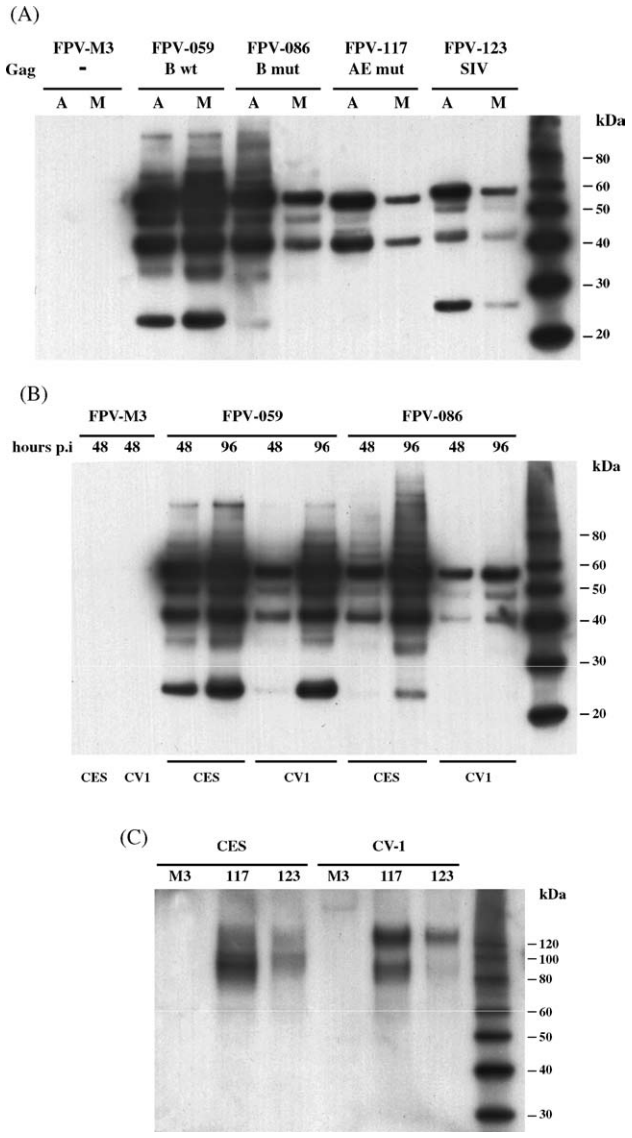

Fig. 2. Western blot analysis of HIV antigen expression from rFPVs. (A) Supernatants from infected CES cells, A, or CV-1 cells, M, 96 h post-infection, probed with anti-p24 MAb183. (B) Supernatants from 48 h and 96 h post-infection probed with anti-p24 MAb183. (C) Supernatants at 96 h p.i. probed with anti-gp120 (recombinant subtype B). CES cell lysates 96 h post-infection probed with: (D) anti-PentaHis (Qiagen); (E) anti-Tat (subtype B); (F) anti-Rev (subtype B).

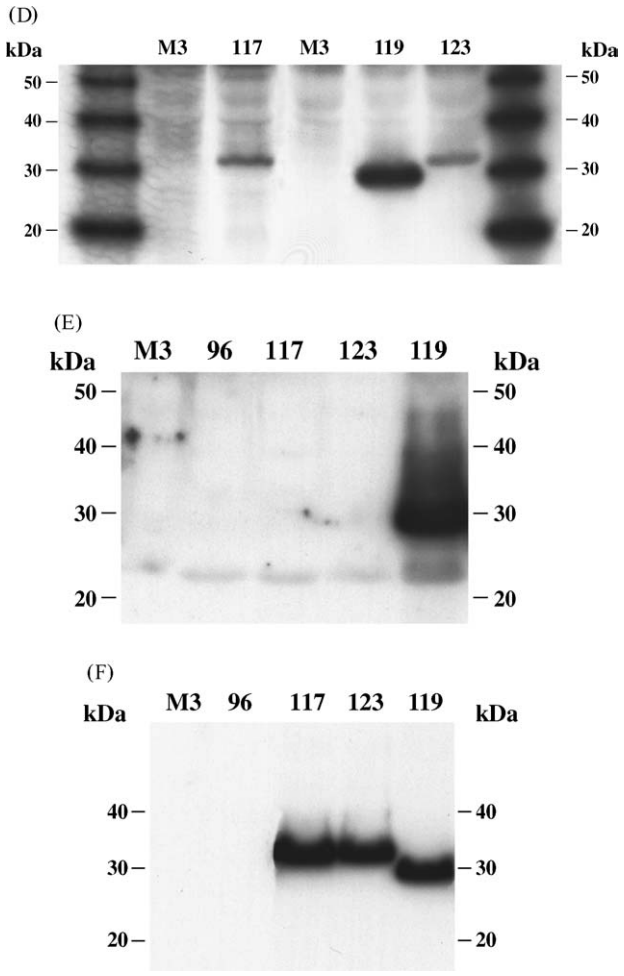

Fig. 2. (*Continued*).

Tat function was assessed by the ability of FPV-expressed Tat or Tat–Rev to induce the expression of GFP in a T cell line transduced with LTR-GFP. Fig. 3 shows data from infections of CEM-LTR-GFP cells with a range of recombinant FPVs. Although the level of FPV infection in this non-avian cell line was extremely low (as detected by anti-FPV serum), GFP expression was stimulated by FPV-096 where the AE version of Tat was expressed without Rev. While levels of fluorescence generated in the presence of rFPV-expressed AE or B Tat–Rev were generally lower than for Tat alone, both versions of the fusion protein where able to interact with the HIV-LTR to induce expression of GFP (Fig. 3). In a separate experiment where plasmids expressing either Tat or Tat–Rev constructs for both AE and B subtypes, using the CMV immediate early promoter, were transfected into a similar T cell line (Jurkat-LTR-EGFP) all four constructs were shown to induce high levels of GFP (data not shown), indicating that the presence of a C-terminal fusion (Rev) did not impede the ability of Tat to interact with the LTR promoter.

Table 3
Quantitation of HIV or SIV Gag expression from rFPVs

Virus[a]	CES cells[b]				CV-1 cells[c]			
	Cell lysate[d]	Supernatant[e]	Total	% Supernatant	Cell lysate	Supernatant	Total	% Supernatant
FPV-059 (B wt)	27,976 ± 736	1403 ± 31	29,379 ± 767	4.77 ± 0.02	48,818 ± 265	1572 ± 6	50,390 ± 259	3.12 ± 0.03
FPV-086 (B mut)	26,773 ± 1372	290 ± 4	27,063 ± 1376	1.07 ± 0.04	9488 ± 51	62 ± 0	9549 ± 51	0.64 ± 0.00
FPV-117 (AE mut)	5751 ± 66	435 ± 3	6186 ± 63	7.03 ± 0.12	12,434 ± 85	147 ± 15	12,581 ± 70	1.17 ± 0.12
FPV-123 (SIV wt)	4673 ± 26	290 ± 4	4,963 ± 22	5.84 ± 0.10	8262 ± 45	51 ± 0	8313 ± 45	0.61 ± 0.00

FPV-M3 control samples all negative.
[a] Infection at m.o.i. 5 pfu/cell for 72 h.
[b] pg of p24 per 10^6 CES cells.
[c] pg of p24 per 10^6 CV-1 cells.
[d] Cell lysates prepared as described in Section 2.
[e] Concentrated supernatants prepared as described in Section 2.

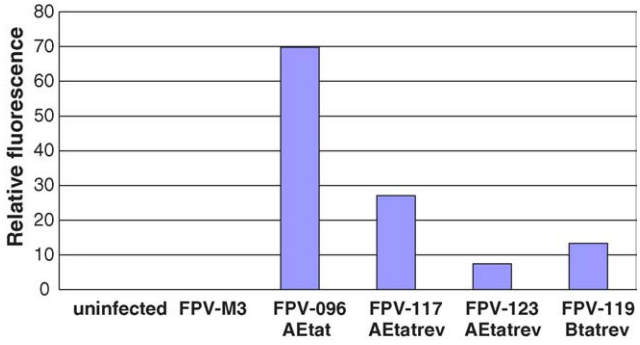

Fig. 3. . Expression of Tat and Tat–Rev fusion proteins from rFPVs. Relative fluorescence (percent positive cells × median channel fluorescence) at 96 h post-infection of CEM-LTR-GFP cells.

3.3. Expression of cytokines from rFPV

Cytokine co-expression by the rFPV vaccines has the potential to provide immunomodulatory activity to enhance vaccine immunogenicity and efficacy. Quantitation of cytokine expression from rFPVs was carried out using ELISA kits specific for each human or murine cytokine (IFN-γ or IL-12) and the data are shown in Table 4. For each of the rFPVs tested cytokine secreted into the culture medium was readily detected after 48 h of infection in CES cells, irrespective of the HIV or SIV antigens co-expressed from the rFPV.

3.4. Immunogenicity of rFPV vaccines in mice

An assessment of the in vivo immunogenicity associated with rFPV-expressed HIV antigens in mice was made by using a DNA prime/rFPV boost vaccination strategy and vaccinia virus challenge (Table 5). Where the challenge virus expressed HIV antigens or epitopes recognised by the mice, VV was cleared to below detectable levels and the mice were considered protected. Fig. 4 shows results of virus titres in ovaries 4 days post-challenge with VV and anti-p24 antibody levels detected in individual mice. In the first experiment (Fig. 4A) animals vaccinated with the matched DNA and FPV vaccines for HIV subtype AE, were protected against VV challenge, provided the challenge virus expressed AE GagPol or Env or Tat–Rev (groups 3–5), but not when non-recombinant VV was used (group 2). When the Tat–Rev insertion in the rFPV was replaced with a murine cytokine (IFN-γ or IL-12) the mice were still protected (groups 6 and 7). Similarly when the second dose of DNA was delivered at week 8 along with the rFPV boost, either mixed at one site (group 8) or DNA and rFPV at separate sites (group 9), mice were able to clear the challenge virus which expressed AE GagPol sequences. In the unvaccinated controls (group 1) four of the seven mice did not survive the VV challenge and blood was not collected from two of the survivors. Although antibody levels were not measured beyond a dilution of 20,480, animals receiving rFPV coexpressing IFN-γ (group 6) did appear to develop higher antibody titres

Table 4
rFPV expression of cytokines

rFPV	Cytokine expressed from rFPV[a]	Additional genes in rFPV (total # insertions in rFPV[b])	Cytokine in culture supernatant (pg/ml)[c]
FPV-087	huIFN-γ	HIV B *gagpol* (2)	94,990
FPV-088	huIL-12	HIV B *gagpol* (2)	238,461
FPV-094	huIFN-γ	SIV *gagpol* (2)	103,588
FPV-100	huIFN-γ	HIV AE *gagpol*, *env* (3)	330,089
FPV-106	muIFN-γ	HIV AE *gagpol*, *env* (3)	25,099
FPV-107	muIL-12	HIV AE *gagpol*, *env* (3)	157,620

[a] Cytokine genes inserted at FPV TK ORF X locus by dominant selection using pAF09 with retention of marker genes *Eco*gpt and β-galactosidase.
[b] Total number of insertions in rFPV including cytokine.
[c] CES cell culture supernants 48 h post rFPV infection, assayed using R&D Systems Quantikine® Immunoassay kits and standards.

Table 5
Mouse treatment groups

Group	Vaccination		Challenge
	Prime (weeks 0 and 4)	Boost (week 8)	VV (week 9)
Experiment 1			
1	Unvaccinated	Unvaccinated	VV-WR[c]
2	DNA-AE[a]	rFPV-AE (FPV-117)[b]	VV-WR
3	DNA-AE	rFPV-AE (FPV-117)	rVV-AE GagPol (VV-336)
4	DNA-AE	rFPV-AE (FPV-117)	rVV-AE Env (VV-337)
5	DNA-AE	rFPV-AE (FPV-117)	rVV-AE TatRev (VV-338)
6	DNA-AE	rFPV-AE + IFN-γ (FPV-106)	rVV-AE GagPol (VV-336)
7	DNA-AE	rFPV-AE + IL-12 (FPV-107)	rVV-AE GagPol (VV-336)
8	DNA-AE (week 0 only)	DNA-AE + rFPV-AE (FPV-117), one site	rVV-AE GagPol (VV-336)
9	DNA-AE (week 0 only)	DNA-AE + rFPV-AE (FPV-117), two sites	rVV-AE GagPol (VV-336)
Experiment 2			
1	Unvaccinated	Unvaccinated	VV-AE GagPol (VV-336)
2	Unvaccinated	Unvaccinated	VV-B GagPol (VVARVTK)
3	DNA-AE	rFPV-AE (FPV-117)	VV-AE GagPol (VV-336)
4	DNA-AE	rFPV-AE (FPV-117)	VV-AE Env (VV-337)
5	DNA-AE	rFPV-AE (FPV-117)	VV-AE TatRev (VV-338)
6	DNA-AE	rFPV-AE (FPV-117)	VV-B GagPol (VVARVTK)

[a] DNA 100 μg IM.
[b] FPV 5×106 pfu IP.
[c] VV 107 pfu IP.

(three of six mice >20,480). Two vaccinated animals (group 5: mouse 3; group 8: mouse 3) with detectable VV titres also had low antibody titres. Since antibody levels were variable across and within groups, and no serum antibodies against HIV-1 Env were detected (data not shown), cell-mediated immune responses in the vaccinated animals would appear to be responsible for the ability to clear the rVV challenge. Further, the ability of vaccinated animals to clear recombinant VV, irrespective of the AE antigen expressed, suggested that the vaccination protocol was sufficient to stimulate T-cell responses to GagPol, Env or Tat–Rev.

To confirm these findings an additional experiment was performed utilising the vaccination regimen outlined in Table 4, and the data are shown in Fig. 4B. Here, the unvaccinated groups were challenged with recombinant viruses (TK negative) rather than the more virulent VV-WR, and the mice in all groups survived. High titres of VV were found in the ovaries of unvaccinated mice (groups 1 and 2), while mice vaccinated with the DNA-AE/rFPV-AE vaccines were able to clear the challenge whether the vaccinia-expressed antigen was AE GagPol (group 3), AE Env (group 4), AE Tat–Rev (group 5) or B GagPol (group 6). Once again antibody responses against the Gag product p24 were variable across the groups and those vaccinated animals with low titres were still able to clear the vaccinia challenge, suggesting a protective cell mediated immune response was in operation.

4. Discussion

HIV vaccines matching circulating viral strains and capable of reliably inducing broad T and B cell immunogenicity, are desperately needed. We constructed several sets of DNA plasmids for priming and rFPV vaccines for boosting immune responses to HIV or SHIV, in a concerted multi-institutional effort to progress these vaccine candidates towards clinical trials. Attempts were made to include as much genetic material from HIV-1 as was possible, within the constraints imposed by safety and regulatory considerations, and to match the sequences included in the rFPV boost to as many as possible of those provided by the priming DNA vaccine. The differences between expression from DNA plasmids, where a single eukaryotic promoter can be used to drive the expression of multiple genes with introns, and from recombinant poxviruses where continuous coding sequences need to be expressed from individual poxvirus promoters, required the construction of complex FPV recombinants with multiple insertions. Here, we describe three sets of rFPV vaccines directed against HIV-1 subtype B, against HIV-1 subtype AE or against SHIV. Importantly for vaccine manufacture, these rFPVs with insertions at up to three locations in the viral genome and including up to five HIV genes, were stable on passage in chick cell culture as determined by PCR analysis (Fig. 1) and by sequencing, and expressed the predicted antigens (Figs. 2 and 3) or co-expressed cytokines (Table 4) in each case.

Safety modifications to the HIV subtype B sequences resulted in reduced levels of processing of the Gag product, p55, to the mature form, p24, particularly when material released from cells in vitro was examined (Fig. 2B). Similarly for the modified HIV subtype AE GagPol (FPV-117) low levels of p24 were observed (Fig. 2A) but without an unmodified version available for comparison. Quantitation of the rFPV-expressed Gag protein showed a reduction in the level released from cells (presumably as pseudoviral particles) in mammalian cell infections, where FPV

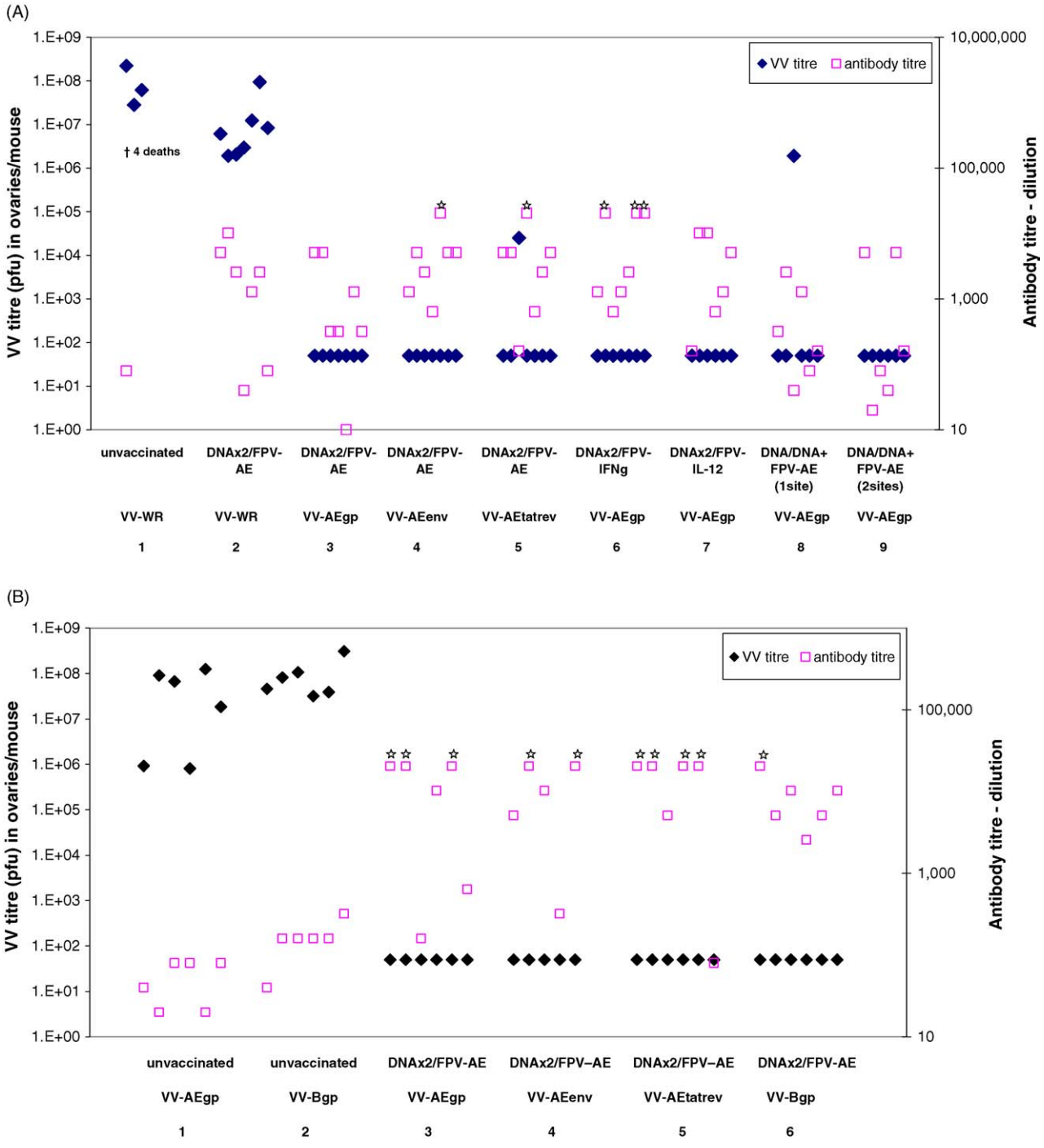

Fig. 4. VV titres (pfu) in ovaries (filled symbols) and anti-p24 antibodies (open symbols) in BALB/c mice post challenge. (A) Experiment 1; (B) Experiment 2. Refer to Table 5 for detailed treatment group descriptions. Antibody titre >20,480. VV titres in all vaccine treatment groups ((A) groups 3–9 and (B) groups 3–6) were significantly different from the unvaccinated groups ((A) group 1 and (B) groups 1 and 2) or group vaccinated and then challenged with VV-WR ((A) group 2) ($p < 0.0005$). There were no significant differences between the vaccine groups except for the group challenged with VV-WR. ELISA antibody titres in the vaccine groups were not significantly different from each other ($p < 0.05$).

undergoes an abortive replication cycle, compared with avian cell infections (Table 3). Differences in the levels of processing between avian and mammalian cell infections were also apparent when the products of a modified AE Env construct were examined by Western blot (Fig. 2C). This highlights the potential trade-off between safety and expression/immunogenicity that is encountered when attempting to induce broadly reactive immunity.

Tat has emerged as a potentially useful HIV-1 vaccine antigen in recent years [20]. The synthetic construct for expression of Tat and Rev as a fusion protein provided a means of expressing both proteins from rFPV using a single

insertion site and with a single promoter, while reducing the likelihood of instability in the rFPV through duplication of sequences. HIV AE Tat and the fusion products AE or B Tat–Rev, expressed from rFPV in the cytoplasm of infected cells, were able to interact with the HIV LTR in the nucleus of transformed cells to induce the production of GFP (Fig. 3). Antisera to HIV-1 subtype B Tat did not react with the subtype AE Tat or Tat–Rev (Fig. 2E), while both forms of Rev were detected using antiserum to subtype B Rev on Western blots (Fig. 2F).

In mice, the immunogenicity afforded by the DNA/rFPV AE vaccines was sufficient to protect against a vaccinia virus challenge, provided the VV expressed HIV sequences (Fig. 4). The lack of correlation between antibody levels and protection suggested that a T-cell response was involved and the ability to clear rVV expressing either HIV AE GagPol or Env or Tat–Rev suggested that T cells specific for each of these genes were involved. In addition, substitution of the rFPV-encoded Tat–Rev sequences with cytokines (IFN-γ or IL-12) proved equally effective against a VV-AE Gag-Pol challenge. Simultaneous delivery of the second dose of DNA priming with the FPV vaccine boost, either mixed at a single site or by separate routes, appeared equally effective and could provide a means of reducing the complexity and enhancing the utility of the vaccine regimen.

Macaque studies involving the use of these rFPV vaccines to boost after DNA priming, have been reported recently for HIV-1 subtype B constructs where the rFPV was used to coexpress IFN-γ or IL-12 [21], for HIV-1 subtype AE where five genes were shared between the DNA and FPV vaccines [22], and for SHIV where efficacy in the face of a virulent SHIV challenge was tested [23]. The HIV-1 subtype B DNA and FPV vaccines described here have been used in an ongoing Phase I/IIa safety and immunogenicity trial in human volunteers in Sydney [Kelleher et al., manuscript in preparation].

In summary, the construction and characterisation of the FPV recombinants reported here provide a robust path towards clinical development of these HIV vaccine strategies. In addition, the development and construction of these complex FPV vectors provide useful indicators for further refinements of the rFPV expression system and its use as a vector for HIV or other antigens in non-avian species.

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