

A recombinant avipoxvirus HIV-1 vaccine expressing interferon-gamma is safe and immunogenic in macaques

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Abstract

Complex recombinant fowlpoxvirus (rFPV) vaccines expressing both HIV-1 antigens and type 1 cytokines could facilitate the induction of cellular immunity against HIV-1. A single rFPV expressing both HIV-1gag/pol and human interferon- γ (FPVgag/pol-IFN γ) was constructed and assessed as a therapeutic vaccine for safety and immunogenicity in macaques (*Macaca nemestrina*) previously infected with HIV-1. FPV gag/pol-IFN γ vaccinations were safe and enhanced T cell proliferative responses to Gag antigens (but not control tetanus antigens). Enhanced CTL responses to gag/pol antigens were also observed following IFN γ expressing vaccinations. Since cellular immunity may be critical to controlling or preventing HIV-1 infection, these observations suggest that avipox vectors co-expressing IFN γ should be further evaluated as therapeutic or preventive HIV-1 vaccines. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Safe and effective preventive or therapeutic HIV-1 vaccines are urgently needed, particularly in developing nations that do not have access to effective drug therapies. Antiretroviral treatments can suppress plasma HIV-1 RNA levels to low levels, however latently infected cells harbouring HIV-1 DNA remain detectable and the development of viral resistance and relapse is common [1]. Treatment-induced reductions in HIV-1 levels result in a loss of antigenic stimulus

for effective immune responses. HIV-specific cytotoxic T lymphocyte (CTL) responses, a critical effector mechanism in the control of HIV-1, decline to low levels following effective anti-HIV therapy [2]. Previous trials of therapeutic HIV-1 vaccines have shown that it is possible to stimulate anti-HIV immune responses in HIV-1 infected individuals, but no clinical benefit has been demonstrated [3]. Prior studies have generally either used protein-based HIV-1 vaccines incapable of inducing CTL responses or vaccinated individuals with substantial levels of replicating HIV-1. Even moderate levels of replicating HIV-1 results in a loss of HIV-specific CD4⁺ T-helper (Th) responses which are required to initiate and sustain effective CTL responses [4,5]. Novel vaccine technologies and approaches are required if therapeutic HIV-1 vaccines are likely to facilitate control of HIV-1.

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Pigtail macaques (*Macaca nemestrina*) are susceptible to acute HIV-1 infection but after a few months only HIV-1 DNA, and not HIV-1 RNA or culturable virus, can be detected and the animals do not progress to immunodeficiency [6]. HIV-specific T cell responses decline to low levels following resolution of acute HIV-1 infection of macaques. Evaluation of the immunogenicity and safety of therapeutic HIV-1 vaccines in pigtail macaques latently infected with HIV-1 could provide safety and immunogenicity information on the potential of therapeutic clinical HIV-1 vaccines.

Recombinant avipoxviruses encoding HIV-1 antigens are promising HIV-1 vaccine candidates [7]. However, avipox-HIV-1 vaccines induce only modest HIV-specific CTL responses in a minority of human subjects [8]. Co-expression of cytokines by recombinant avipox vaccines can dramatically modulate the resulting immune responses in mice, but this approach has not been assessed in non-human primates [9]. Interferon-gamma ($\text{IFN}\gamma$) is a cytokine critical in the induction of CTL responses and type 1 Th responses (Th1, Th cells that secrete IL-2 and $\text{IFN}\gamma$). A recombinant fowlpoxvirus expressing $\text{IFN}\gamma$ and HIV-1Gag/Pol was constructed and assessed for safety and immunogenicity as a therapeutic vaccine in macaques previously infected with HIV-1.

2. Materials and methods

2.1. Vaccines

The HIV-1gag/pol genes of ARV-2/SF2 strain with or without the human $\text{IFN}\gamma$ gene were inserted into the FPV genome (FPV M3 strain) along with the *E. coli* β -gal and/or *gpt* selection and marker genes [10]. The insertion was made between the FPV *thymidine kinase* gene and the immediately downstream 3' open reading frame. For the FPVgag/pol recombinant, the gag/pol genes were placed under the control of the FPV P.E/L bi-directional promoter, aligned so that the native start codon of the gag gene is utilised for translation to ensure optimal expression and myristylation of Gag proteins [11]. For the FPVgag/pol- $\text{IFN}\gamma$ recombinant, the $\text{IFN}\gamma$ gene was cloned by PCR into the FPV plasmid vector, pAF09, under the control of the P.E/L promoter. The gag/pol genes under control of the vaccinia virus P.7.5 promoter were then cloned immediately downstream of $\text{IFN}\gamma$. Recombinant FPVs were constructed using established methods for selection based upon the co-expression of β -gal and/or *gpt*. After plaque purification, the construction and homogeneity of the recombinants were confirmed by PCR analysis using primers complementary to the sequences flanking the insertion site and the inserted genes. In vitro, FPVgag/pol- $\text{IFN}\gamma$ expresses both gag/pol anti-

gens and $\text{IFN}\gamma$ as assessed by immunoblot, ELISA and functional assays (data not shown).

2.2. Animals and vaccinations

M. nemestrina were anaesthetised with Ketamine prior to procedures. The studies were approved by the Institutional Animal Ethics Committees. Seven animals were studied that have been previously described [6,11]. Three animals (M7, M9, M10) had no previous HIV-1 vaccinations and were infected with HIV-1 following an intravenous challenge with HIV-1_{LAI} 9 months prior to this study. Two animals (M9, M10) received a FPV expressing Gag/Pol and human $\text{IFN}\gamma$ and one animal (M7) received a FPV vaccine expressing Gag/Pol only. Each FPV vaccine was given IM at 10^8 PFU in 0.3 ml at 9 and 12 months following HIV-1 infection to the right anterior thigh. Four animals (M2, M3, M4, M5) served as controls and received no vaccines during the course of this study. These 4 animals (M2–5) had been vaccinated with DNA and FPV HIV-1 vaccines (not containing cytokines) 11–19 months prior to study and resisted a HIV-1 challenge 9 months prior to this study [11]. All 7 macaques were previously vaccinated with three doses of tetanus toxoid (CSL, Parkville, Australia) prior to HIV-1 vaccinations.

2.3. Safety assessments

Animals were assessed twice daily following vaccination for visible swelling and redness, characterised as either limited to the site of injection (anterior thigh), involving the whole thigh, or the whole limb. Activity of the macaques was monitored by counting normal macaque behaviours as described [12]. Briefly, interval sampling of foraging, grooming, displacement, mounting and individual and conspecific paly activities were recorded for 15 min daily and total behaviour events recorded. Mean behaviour scores taken for 1 week prior to vaccination were compared those following vaccinations for 6 weeks. A 25% reduction in total activity compared with the mean baseline activity in the week prior to vaccination was considered significant. Temperature recordings were determined by using an electronic thermometer (Braun Thermoscan) and training the animals to have this applied to their tongues. This method of taking temperatures was 0–0.8°C (mean 0.3°C) lower than rectal temperatures taken on sedated macaques on 22 consecutive occasions. Biochemical analyses and blood counts were performed on automated machines and counts confirmed manually. PBMC were analysed by flow cytometry using antibodies directed against CD4, CD2, CD8, and CD14 as previously described [6].

2.4. HIV-1 antibody and Th responses

Plasma was assessed for HIV-1 antibodies by particle agglutination (Serodia-HIV, Fujirebio, Japan) and Western blotting using 200 µg of mixed HIV-1 protein stock [6]. Lymphoproliferative responses were assessed by ³H-thymidine incorporation as described [6]. Briefly, PBMC were stimulated for 6 days with 10 µg/ml of rHIV-1_{SF2} gp120, rHIV-1_{SF2} p24 (Chiron), tetanus toxoid antigen (0.01 Lf/ml) or control antigens in media containing 10% autologous heat-inactivated serum and pulsed with ³H-thymidine before β-counting. Proliferation is expressed as stimulation index (SI, mean ³H-thymidine incorporation of cells stimulated with antigen/mean incorporation without stimulation). Lymphoproliferative culture supernatants were assayed for IL-4 and IFNγ by EIA (Genzyme, Cambridge, MA).

2.5. Quantitative HIV-specific CTL analyses

Analysis of CTL precursor frequencies to HIV-1 Env and Gag/Pol antigens in macaque PBMC of macaques was performed by a limiting dilution analysis [6]. Briefly, PBMC were plated in seven serial dilutions of 10⁵ to 8.8 × 10³ cells/well in 24 replicates and stimulated with 10⁴ autologous PBMC infected with a recombinant vaccinia virus expressing HIV-1_{LAI}Env/Gag/Pol antigens and 10 U/ml rIL-2 (Hoffman-La Roche, Nutley, NJ). After 10 days, cells in each well were assayed for cytolytic activity against autologous target cells infected with wild type vaccinia or recombinant vaccinia expressing HIV-1_{LAI}Env antigens or HIV-1_{LAI}Gag/Pol [6]. Wells were considered positive if cytolysis exceeded mean spontaneous release by 3 SD. CTL frequencies and 95% confidence intervals were determined by maximum likelihood analysis [6]. Targets were autologous B lymphoblastoid cell lines, established by infecting PBMC with *H. papio*, a baboon herpesvirus [6]. B lymphoblastoid cell lines could not be transformed from PBMC of one control animal (M4) and could not be maintained in long term culture from one vaccinated animal (M10) and CTL data could not be generated from those animals.

2.6. Analysis of HIV-1 DNA, RNA and culturable virus

HIV-1env and HLA-DQ DNA were amplified from extracted PBMC DNA and quantified using primer pairs SK68/69 and GH26/27 as previously described [6], with a limit of detection of 1–3 HIV-1 DNA copies/10⁵ cells. Virus isolation was performed by cocultivation of 10⁶ macaque PBMC with 10⁶ PHA-stimulated human PBMCs [6]. Plasma HIV-1 RNA was assessed by RT-PCR (Amplicor, Roche, Branchburg, NJ), limit of detection 20 copies/ml.

3. Results

3.1. Safety of FPV expressing IFNγ

Locally delivered cytokines encoded by viral vectors are generally less toxic than systemically administered cytokines [9]. We analysed the reactogenicity of FPVgag/pol-IFNγ in comparison to a control animal receiving FPVgag/pol only and 4 unimmunised age-matched controls as a therapeutic vaccine strategy following prior HIV-1 infection. A high dose of the FPV vaccines was administered (10⁸ PFU) in an attempt to detect any adverse effects. There was no apparent difference in the reactogenicity of FPVgag/pol and FPVgag/pol-IFNγ vaccinations. A 44–75% reduction in activity of all 3 FPV-immunised macaques was observed for 24 h following vaccination, and in one FPVgag/pol-IFNγ immunised animal (M9), 28% reduction of activity was present between 24–48 h, but was normal thereafter in all animals. The reduction in activity observed following FPV vaccination was similar in magnitude to that seen in macaques following a tetanus vaccination, with a 14–82% (mean 42%) reduction in activity scores detected during the first 24 h observed following prior tetanus vaccination in the seven pigtail macaques studied.

Swelling limited to the injection site of the right anterior thigh was observed for 1–2 days following vaccinations in all three FPV vaccinated animals. This was similar to that observed following tetanus toxoid vaccinations of the seven animals studied, also demonstrating transient localised swellings. No fever was documented following the FPV vaccinations (Fig. 1A). All animals gained weight normally (not shown). No change in CD4+ or CD8+ T cell subsets, or monocyte levels in PBMC were observed following vaccination by (Fig. 1B). No significant changes in plasma electrolytes, liver function markers, haemoglobin, white cell or platelet counts were observed following FPVgag/pol or FPVgag/pol-IFNγ vaccination (not shown).

3.2. T cell immunogenicity

To determine whether vaccination with FPVgag/pol-IFNγ enhanced Gag/Pol specific Th responses, macaques infected with HIV-1 9 months previously were vaccinated twice with FPVgag/pol-IFNγ (2 animals, M9 and M10) or FPVgag/pol (1 animal, M7). Th proliferative response to p24 Gag protein was enhanced 4–7-fold 1–2 weeks after the first FPVgag/pol-IFNγ vaccination and was greater than baseline levels 3 months later (Fig. 2A). Following a second FPVgag/pol-IFNγ vaccination, p24-specific Th responses were further boosted above baseline (5–30-fold) and maintained for at least a further 2 months. The animal

which received 2 FPVgag/pol immunisations had a 3-fold enhancement of the p24-specific Th response. Tetanus-specific Th responses did not change following FPV vaccinations (<3-fold variation over time). The Th responses to Gag or tetanus antigens of four control macaques (M2, M3, M4, M5) did not change, with a <2-fold variation over the 4 month observation period (mean SI to p24 was 3.2 and to Tetanus toxoid 3.6).

We also assessed whether FPVgag/pol-IFN γ vaccination of HIV-1 infected animals was associated with a change in the phenotype of Gag-specific Th responses. Enhanced IFN γ secretion, but not IL-4 secretion, by Gag-specific Th responses from PBMC of animals receiving both FPVgag/pol and FPVgag/pol-IFN γ was observed, with the magnitude of the modu-

lation of the cytokine secretion being greater in the FPVgag/pol-IFN γ immunised animals (Fig. 2B). No change in the tetanus-specific Th phenotype from animals M7, M9, and M10 was observed following FPV vaccinations, with IL-4 secretion exceeding that of IFN γ (by 4–12-fold) both before and 2–6 weeks after FPV vaccinations of all three FPV vaccinated animals.

3.3. HIV-specific CTL activity following FPVgag/pol-IFN γ immunisation

Considerable interest currently focuses on immunisation strategies to maintain CTL responses despite a reduction in antigenic stimulus from replicating HIV-1 [2,6]. HIV-1 specific CTL response in macaques parallel the reduction in HIV-1 DNA following the first few

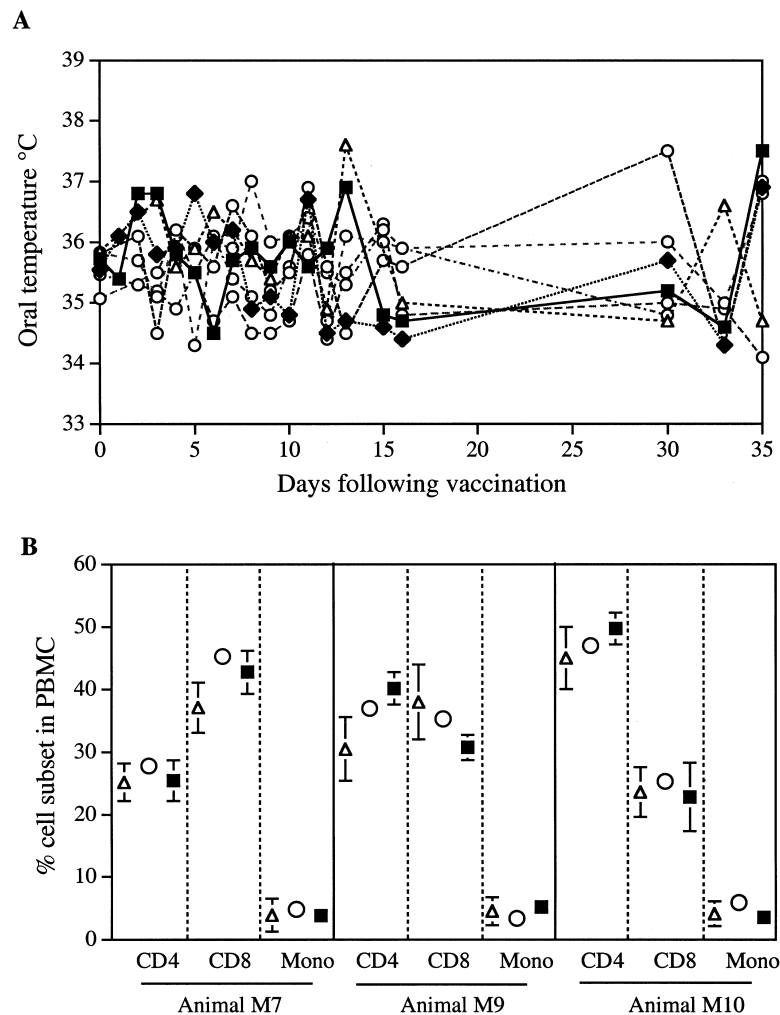


Fig. 1. Safety of FPVgag/pol-IFN γ immunisation. (A) No significant fever was documented following FPVgag/pol-IFN γ vaccination of macaques. Animals M9 and M10 (◆, ■) received FPVgag/pol-IFN γ 10⁸ PFU IM, animal M7 (Δ) received FPVgag/pol, and animals M2, M3, M4, and M5 (○) were unvaccinated controls. (B) No change in T cell or monocyte counts was observed following FPVgag/pol-IFN γ vaccination of macaques. PBMC obtained from animals vaccinated with FPVgag/pol-IFN γ (M9, M10) or FPVgag/pol (M7) were assessed for CD4+2+ T lymphocytes, CD8+2+ T lymphocytes and CD14+ monocytes prior to vaccination (Δ, 6 times over 8 months prior to vaccination, mean \pm SD shown), on the day of vaccination (○) and following vaccination (■, weekly for 4 weeks following vaccination, mean \pm SD shown).

months of HIV-1 infection, and in the “latent” phase HIV-1 specific CTL responses are low (≤ 10 HIV specific CTLs/ 10^6 PBMC) [6]. By a limiting dilution analysis, CTL precursors to Gag/Pol (but not Env) antigens were enhanced from < 5 to $15/10^6$ PBMC following one FPVgag/pol-IFN γ vaccination and to $44/10^6$ PBMC following a second FPVgag/pol-IFN γ vaccination (Fig. 3). Gag/Pol or Env specific CTLs were not detectably enhanced (remaining $\leq 5/10^6$ PBMC) in controls animals either unvaccinated (M2, M3, M5) or vaccinated with FPVgag/pol without IFN γ (M7, Fig. 3).

3.4. HIV-1 antibody levels

Gag/Pol specific antibodies were also enhanced following the two FPV vaccinations (Fig. 4). p24-specific

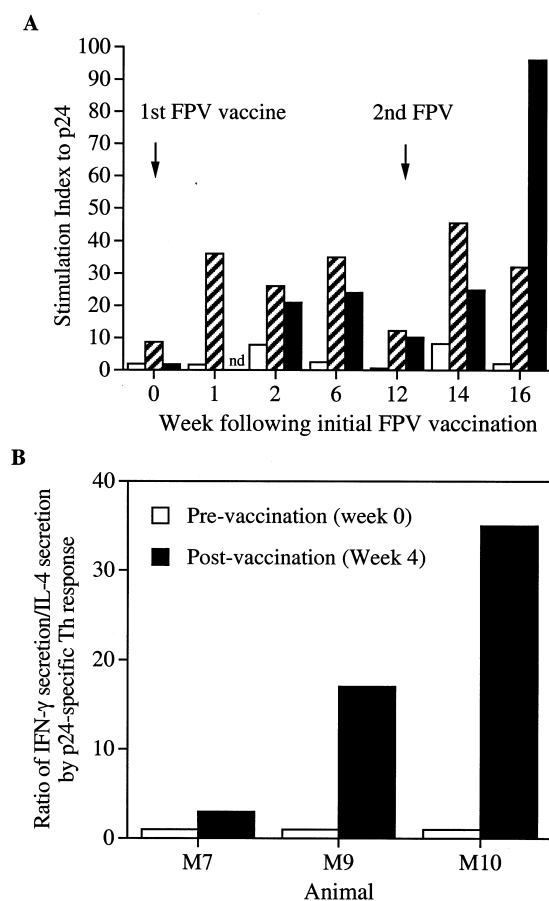


Fig. 2. Enhanced gag-specific Th1 response following FPVgag/pol-IFN γ vaccination of macaques. (A) T cell proliferative response to p24 antigen was measured serially following two vaccinations (arrows) of macaques with FPVgag/pol-IFN γ (animals M9, M10, solid and hatched bars) or FPVgag/pol vaccinations of macaques (animal M7, open bars).

(B) Secretion of IFN γ and IL-4 by PBMC in response to recombinant HIV-1_{SF2}p24 protein stimulation obtained before and after the first FPV vaccination. FPVgag/pol-IFN γ vaccinated macaques (M9 and M10) and a FPVgag/pol immunised animal (M7) are shown.

antibodies were enhanced in all three vaccinated animals, with no difference observed between the FPVgag/pol-IFN γ and FPVgag/pol vaccinated animals. No change in gp120 antibody responses was observed.

3.5. HIV-1 levels following vaccination

To determine whether FPVgag/pol-IFN γ vaccination altered HIV-1 viral levels in macaques previously infected with HIV-1, HIV-1 DNA and culturable virus were studied before and after vaccinations. Using *env*-specific primers, animals M7, M9 and M10 had ≤ 10 copies of HIV-1 DNA/ 10^5 PBMC 0, 1, and 4 months prior to vaccinations and remained at ≤ 10 copies of HIV-1 DNA/ 10^5 PBMC at 1, 2 and 4 weeks following the first FPV vaccination and 2 and 4 weeks following the second FPV vaccination, without detectable changes (< 3 -fold variation) in HIV-1 DNA levels. HIV-1 could not be recovered from PBMC of the three vaccinated FPV animals either prior to (weeks 0, -4) or following first (weeks +1, +2, +4, +6) and second (weeks +2, +4) vaccination. Plasma HIV-1 RNA could not be detected (< 20 copies/ml) 1–4 weeks following the first and second FPV vaccinations in any animal except for animal M9 which had 90 copies/ml of HIV-1 RNA detected 1 week following the first FPV vaccination but had undetectable HIV-1 RNA at 2 and 4 weeks following the first and second vaccinations.

4. Discussion

This study demonstrates that vaccination with FPVgag/pol-IFN γ is safe and immunogenic in non-human primates. Transient reductions in macaque activity were observed, but no fever, haematological,

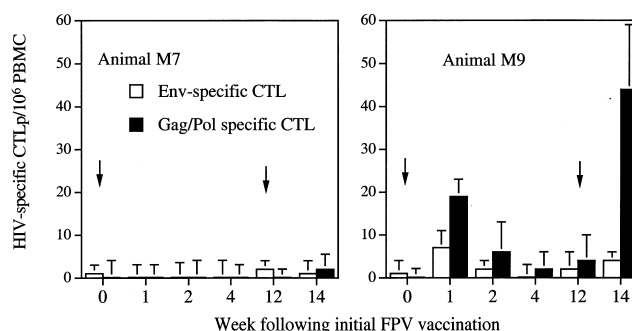


Fig. 3. Enhanced Gag/pol specific CTL response following FPVgag/pol-IFN γ vaccination. Quantification of CTL precursors to Env and Gag/pol antigens was analysed following FPV vaccinations. CTL frequencies were assessed following initial and booster FPV vaccinations (arrows). Recognition of targets expressing vaccinia antigens alone have been subtracted.

or biochemical abnormalities were detected. Enhanced HIV-specific Th and CTL responses were detected, reaching levels equal to or (in the case of the Th response) greater than levels previously shown to correlate both with the control of acute HIV-1 infection and protective immunity from HIV-1 in macaques [6,11]. Consistent with the pro-Th1 nature of $\text{IFN}\gamma$, the cytokine profile of the Gag-specific Th responses generated by this vaccine strategy was Th1, believed to be required for effective HIV-1 vaccine strategies. The ability to safely and efficiently modulate cellular immune responses in non-human primates with vaccines cytokine co-expressing offers further hope that a safe and effective HIV-1 vaccine is possible.

The phenotype and magnitude of the HIV-specific T cell responses was modulated by FPVgag/pol- $\text{IFN}\gamma$ vaccination, presumably by the local action of $\text{IFN}\gamma$ on naïve T cells during priming. It remains to be determined, however, whether FPVgag/pol- $\text{IFN}\gamma$ vaccination results in an expansion of the T cell repertoire directed towards Gag/Pol, or only enhances levels of existing Gag/Pol specific T cell responses previously induced by HIV-1 infection. Interestingly, Gag/Pol antibodies were boosted by FPVgag/pol- $\text{IFN}\gamma$ vaccinations, without being modulated by the $\text{IFN}\gamma$ expression in comparison to FPVgag/pol vaccinations

without the $\text{IFN}\gamma$. This could reflect the priming effect of the prior HIV-1 infection.

Other vaccine strategies using poxviruses and cytokine adjuvants have been studied. In a recent SIV/macaque study, IL-12 or IL-2 was delivered in a separate vaccinia vector [13]. Partial protection from virulent SIV challenge observed in macaques receiving vaccines either with or without the cytokines. Although single poxvirus vectors expressing both cytokines and antigens are more difficult to construct, murine models demonstrate that encoding cytokines within the same vector also expressing the vaccine antigens is more efficient at modulating immunity than delivering the cytokine in a separate poxvirus vector [14]. DNA vaccines co-expressing cytokines have been reported to be immunogenic in non-human primates [15]. Concerns about toxicity from long-term expression of cytokines by DNA vaccines may eventually limit this approach in comparison to poxvirus vector approaches, which would be expected to be cleared [16].

Although the primary goal of this study was to assess the safety and immunogenicity of the $\text{IFN}\gamma$ expressing FPV, further interpretation of the results of this study is limited by the small numbers of macaques vaccinated available for study and the non-pathogenic model analysed. A number of efforts were made to

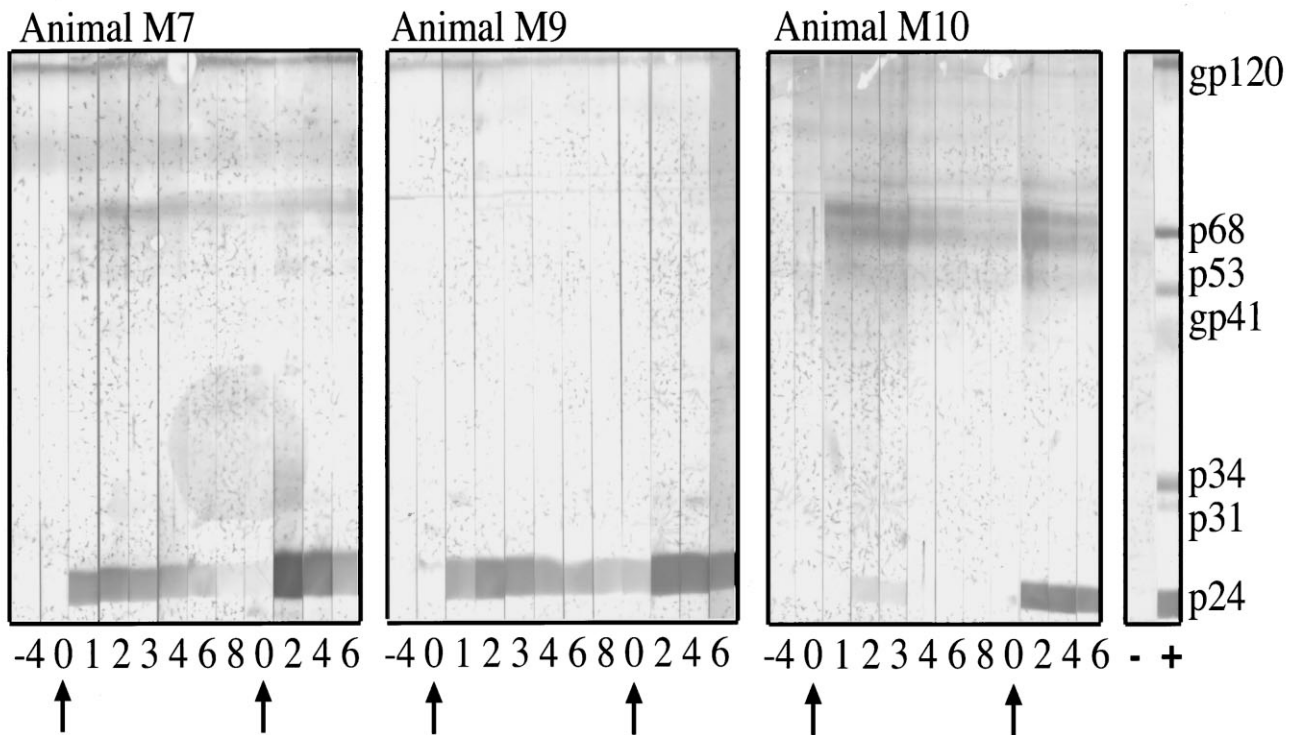


Fig. 4. Gag/Pol specific antibodies are enhanced following FPVgag/pol vaccination. Western blotting of serial plasma (1:100 dilution) from animals M7, M9 and M10 following FPV vaccinations (arrows). Strips are labelled with weeks prior to or following the first and second vaccinations. Negative and positive controls represent uninfected and HIV-1 infected humans respectively.

overcome some of the limitations associated with analysis of small numbers of valuable primate subjects. Multiple vaccinations were administered, an analysis of immune responses to control non-vaccine HIV-1 antigens (Env) and irrelevant non-HIV-1 antigens (Tetanus) was undertaken and we studied a number of control unvaccinated animals with prior HIV-1 specific immunity (animals M2–5). All of these analyses suggested that the FPV-HIV vaccines studied were immunogenic and safe in this model. In future studies, larger numbers of lentivirus-naïve macaques will be immunised with IFN γ expressing FPV following DNA vaccination to further assess the immunogenicity of this approach as a preventive vaccine. A limitation of this pigtail HIV model studied is that, although no substantial virologic effect on HIV-1 DNA, RNA or culturable HIV-1 was detected, pigtail macaques are not permissive to high levels of HIV-1 replication and the model is therefore not suited to detecting large changes in viral replication. This presumably reflects both constitutive factors which limit high level HIV-1 replication in macaques, and HIV-1 specific immune responses [6]. It is possible that the low level of HIV-1 replication (detectable HIV-1 RNA) observed in one macaque following immunisation may have also contributed to the HIV-1 specific T cell immunogenicity observed.

The low and unchanged levels of HIV-1 DNA observed prior to and following vaccinations may represent a latent pool of HIV-1 infected cells not easily amenable to immune or antiretroviral control [17]. Vaccine studies on macaques with SIV replication suppressed by antiretroviral therapy would better address modulation of virologic control by therapeutic vaccines. However, long term pharmacological control of SIV replication in macaques would be technically difficult and such SIV studies alone could not assess the safety or immunogenicity of HIV-1 gag/pol vaccines suitable for future human application.

In conclusion, our results suggest that recombinant poxviruses co-expressing cytokines are safe and immunogenic in macaques and warrant further study both as preventive and therapeutic HIV-1 vaccines.

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