

Research Paper

# Randomised, Placebo-Controlled, Phase I/IIa Evaluation of the Safety and Immunogenicity of Fowlpox Virus Expressing HIV gag-pol and Interferon- $\gamma$ in HIV-1 Infected Subjects

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## ABSTRACT

We conducted a randomised, placebo-controlled double-blind trial to examine the safety and immunogenicity of a candidate HIV therapeutic vaccine based upon a recombinant fowl pox virus capable of coexpressing the human cytokine interferon-gamma and/or genes from HIV-1. Thirty-five eligible subjects were randomised (12 placebo, 11 fowlpox + HIV genes, 12 fowl pox + HIV genes + interferon gamma). All but one subject (placebo group) received three immunizations (by intramuscular injection on day 0, week 4 and week 12) and all completed 52 weeks of follow-up. All subjects continued to take combination antiretroviral therapy for the duration of study. There were no significant toxicity or safety concerns and the distribution of adverse events and their severity was consistent across each randomly assigned vaccine group. Comparison of placebo recipients with the combined recipients of the two vaccine constructs, in terms of anti-HIV gag ELISpot or lymphoproliferative responses, tended to favour the placebo group, but were not significantly different (difference in time-weighted mean change from baseline = 56 Spot forming units (sfu)/10<sup>6</sup> PBMC;  $p = 0.062$  and 4.4 SI;  $p = 0.337$ ). There were no significant changes in CTL responses by standard Cr<sup>51</sup> release assay. Anti-FPV antibodies were detected by week 14 in 0 placebo and 20 (87%) vaccine recipients. Although safe, neither vaccine construct appeared to possess detectable T-cell mediated anti-HIV immunogenic properties in HIV infected individuals, as measured by standard T cell assays.

## INTRODUCTION

The development of therapeutic vaccines for HIV disease has been a feature of clinical research since the mid 1980's. Conceptually, therapeutic vaccination requires new immune responses or enhancement of existing immune responses to occur in an individual following vaccination. It is further postulated that these immune responses will reduce HIV replication. Early candidate vaccines did not satisfy criteria for further development or licensure.<sup>1-4</sup> Many of these candidate vaccines were able to induce demonstrable (albeit inconsistently and at relatively low levels) immune responses of either B-cell or T-cell origin. These observations emphasise the poor understanding of the protective correlates of anti-HIV immunity. Data suggest that cytotoxic T lymphocytes play a key role in controlling HIV replication during primary infection and perhaps in certain types of more chronic disease.<sup>5-9</sup> From these observations new types of candidate HIV vaccine have been developed with the specific objective of inducing T-cell mediated anti-HIV immune responses.<sup>10,11</sup> Live virus vector vaccines (such as recombinant pox virus) encoding HIV genes with or without human cytokines are one such example with defined immunogenicity in nonhuman primate models of HIV infection and other chronic virus diseases in humans.<sup>12-15</sup> The development of a safe and effective therapeutic vaccine for HIV could result in a number of significant changes to the care of people with HIV. Control of HIV replication could extend the interval in which patients might defer using expensive and potentially toxic antiretroviral therapy. Similarly, patients who have exhausted viable treatment options with combination ARV might benefit from the use of a vaccine that would forestall further disease progression until more viable therapies are developed. These goals are idealistic, further challenges to vaccine development relate to whether or not vaccine induced immune responses would be robust and sustainable. In addition, induction of immune responses in HIV infected individuals to widely available public health vaccines are known to be sub-optimal and this may further limit the utility of therapeutic vaccines. This is

particularly the case in patients with ongoing HIV replication and advanced immunodeficiency. Given the genetic diversity of HIV it is also likely that vaccines will need to be developed that generate immune responses to the predominant circulating HIV clade within a community or perhaps to generate immune responses to HIV epitopes that are not subject to diversity.

Poxviruses multiply in the cytoplasm of permissive avian host cells, display intragenic genetic recombination and participate in nongenetic reactivation.<sup>16</sup> In human cells avian pox viruses undergo an abortive replication cycle in which only part of the genome is transcribed and translated. Poxvirus recombinants offer several advantages over other replicating virus vectors—including the ability to accommodate foreign genes of up to 25 kb while maintaining genome stability.<sup>17</sup> Other advantages include heat stability, ease of administration and the ability to stimulate both cell-mediated and antibody immunity of relatively long duration.<sup>13-15</sup>

Recombinant fowlpox virus vector vaccines were designed and constructed as previously described to deliver a combination of HIV-1 *gag/pol* with and without the pro-inflammatory cytokine human interferon- $\gamma$  gene intramuscularly into HIV-1 infected individuals.<sup>12</sup> The *gag/pol* genes of HIV-1, isolate ARV-2/SF2 (corresponding to nucleotides 684-5132, Genbank Accession No K02007) were excised from an existing plasmid (pUC19.ARV (Chiron Corporation, Emeryville CA, USA). These were then used for the multi-step synthesis of the fowl pox vectors used in this trial. These HIV genes were selected since proteins expressed from them encompass the known conserved regions of the HIV genome as well as many well characterised T-cell epitopes. HIV SF2 is a prototypic clade B strain and was selected on the basis that the predominant circulating clinical strain in Australia is also clade B.

IFN- $\gamma$  is secreted primarily by T cells, induces activation of macrophages and exerts wide ranging antimicrobial effects against many nonviral pathogens.<sup>18</sup> Several trials have been conducted to determine the safety and tolerability of IFN- $\gamma$  in HIV-positive patients.<sup>19-21</sup> Inclusion of this cytokine was based upon its likely capacity to reverse the relative defects in major histocompatibility complex class II expression, induce HIV-specific CD4<sup>+</sup> T cell responses and induce interleukin-12 production. Inclusion of the cytokine within the same expression system as the HIV genes is designed to result in a self-adjuvanting vaccine. Given the relatively small and localised amounts of cytokine produced it was hoped the inherent problems of exogenous cytokine administration would be avoided.

Both humoral and cell-mediated responses were induced against HIV-1 *gag/pol* epitopes in preclinical studies conducted in mice and nonhuman primates vaccinated with a fowlpox virus vector expressing HIV-1 *gag/pol* plus and minus IFN- $\gamma$ . Enhanced T cell proliferative and CTL responses to gag antigens were observed in macaques administered recombinant fowlpox coexpressing *gag/pol* and IFN- $\gamma$  relative to vector expressing *gag/pol* alone. Response levels were equal to or in the case of the proliferative 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.<sup>12</sup> Cell lines infected with a recombinant fowlpox virus encoding *gag/pol* and IFN- $\gamma$  produced 200-units/10<sup>6</sup> cells/day of IFN- $\gamma$ .<sup>22</sup> No evidence of toxicity was observed in either system.

The objective of the current study was to assess the safety and immunogenicity of two candidate therapeutic vaccines—one expressing HIV *gag/pol* sequences the other coexpressing the HIV *gag/pol* sequences and human interferon- $\gamma$ . Although immune

competence is partially reconstituted during HAART,<sup>23,24</sup> HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell immune responses decrease with time on therapy.<sup>25-28</sup> Therefore, we reasoned that the population of HIV-infected patients most likely to mount detectable anti-HIV immune responses following administration of the vaccines were those treated either during or shortly after primary HIV infection. It was hypothesized that vaccination would enhance anti-HIV immune responses. In addition, we proposed that coexpression of IFN- $\gamma$  would enhance such responses even further.

## METHODS

**Synthesis and preclinical testing of the vaccine constructs.** Candidate vaccines were designed manufactured and underwent preclinical characterization for safety and immunogenicity as previously described.<sup>12,29</sup>

**Patient eligibility and recruitment.** Patients were recruited through a network of eight investigational sites in Sydney and Melbourne representing both primary and tertiary care facilities. Adult patients were eligible if HIV infection was demonstrated by licensed diagnostic procedures and if they had been treated with combination antiretroviral therapy (ART) within six months of primary infection. Patients must have received minimum triple ART for at least six months; maintained CD4<sup>+</sup> counts above 400 cells/ $\mu$ L except for a maximum of up to four consecutive weeks; had experienced undetectable plasma virus load (pVL) (< 400 copies/mL) for the duration of their exposure to ART, following their initial response. Patients had stabilized on their current regimen for a minimum of six weeks and presented with safety variables within clinically acceptable ranges [haemoglobin > 95 g/L, granulocytes > 1.5 x 10<sup>9</sup>/L, platelets > 100 x 10<sup>9</sup>/L, ALT/AST < 5 x upper limit of normal (ULN) and creatinine < 0.16 mmol/L]. All patients were asked to provide written informed consent approved by participant site Research Ethics Committees.

**Clinical trial protocol and study design.** In this double blind, randomized, placebo-controlled study, eligible patients were randomly allocated to receive one of three vaccines, as follows;

- I. Vaccine diluent alone (n = 12) - Placebo Group
- II. Recombinant fowlpox expressing HIV *gag/pol* (n = 12) - Partial Construct Group (PC)
- III. Recombinant fowlpox expressing HIV *gag/pol* and human IFN- $\gamma$  (n = 12) - Full Construct Group (FC)

Candidate vaccines and placebo (diluent alone) vaccines were administered by intramuscular injection in the deltoid muscle on day 0, week 4 and week 12 at a unit dose of 5 x 10<sup>7</sup> plaque forming units per mL (pfu/mL) in phosphate-buffered saline (PBS), 10% glycerol (1.0 mL total volume). These vaccinations were administered in outpatient units of major teaching hospitals and patients were observed for at least one hour after injection. Patients were required to continue combination antiretroviral therapy for the duration of the study.

**Study schedule.** During the study, patients were required to attend for frequent clinical assessment and blood sampling. A total of nine assessment points were included over the first 26 weeks. There were two further assessment points (week 40 and 52) primarily intended as a safety follow-up. No immunological assays were performed after week 26.

**Safety parameters.** Safety assessments (that included clinical examination, HIV plasma viral load, biochemistry and haematology) were performed at week -8, 0, 1, 4, 6, 12, 14, 26, 40 and 52.

**T-cell subset enumeration.** T-cell subsets (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> absolute numbers and percentage, CD4<sup>+</sup>/CD8<sup>+</sup> ratio) were enumerated at week -2, 0, 6, 14 and 26.

**Cytotoxic T lymphocyte assays.** Lymphoblastoid cell lines (B-LCL) were established for each patient, by transforming peripheral blood mononuclear cells (PBMC) with Epstein-Barr virus (from the culture supernatant of the Marmoset cell line, B95.8). Cytotoxic T lymphocyte assays were conducted using PBMC at weeks -2, 0, 6, 14 and 26. PBMC were isolated from heparinized venous blood and one fifth of these were stimulated with PHA (10  $\mu$ g/mL) at a concentration of 2 x 10<sup>6</sup> cells/mL for 24 hours

Table 1 Selected baseline demographics and clinical characteristics

	Placebo	Partial Construct	Full Construct
n	12	11	12
% Male	100	100	100
% Caucasian	83.3	81.8	100
Age - mean $\pm$ SD (years)	38.1 $\pm$ 5.0	37.5 $\pm$ 7.5	40.9 $\pm$ 4.2
CD4 <sup>+</sup> cell count-mean $\pm$ SD (cells/ $\mu$ L)	929 $\pm$ 299	966 $\pm$ 333	970 $\pm$ 281
Percentage patients with undetectable (< 50 copies/ml) plasma HIV RNA	100	100	100
Duration of ART - mean $\pm$ SD (months)	41.2 $\pm$ 22.4	40.7 $\pm$ 20.4	48.2 $\pm$ 20.7
<b>Percentage ever received</b>			
NRTI	100	100	100
NNRTI	41.7	72.7	33.3
PI	91.7	81.8	100
<b>Percentage currently receiving</b>			
NRTI	91.7	63.6	83.3
NNRTI	33.3	54.5	8.3
PI	66.7	54.5	91.7

at 37°C in RPMI 1640 (Trace Scientific, Noble Park, Australia) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100  $\mu$ g/mL streptomycin and 100 U/mL penicillin. Stimulated cells were washed and combined with the unstimulated PBMC in RPMI 1640, 10% FCS, 10 U/mL recombinant IL-2 at 37°C for 6 days.

Autologous B-LCL targets were prepared by incubating 2 x 10<sup>6</sup> B-LCL with Vac.lac VSC8 (NIH AIDS Reagent Program), Vac.gag ADP (Medical Research Council AIDS Reagent Program), Vac.pol VVTG 3167 or Vac.nef VVTG (Transgene, Strasbourg, France) for 90 minutes at 37°C and then labelled with 100  $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham Biosciences Corp, Piscataway, NJ, USA) overnight.

Effector and target cells were washed twice, resuspended in RPMI 1640, 10% FCS and plated at 5 x 10<sup>3</sup> cells per well of a 96-well, U-bottom plate at effector to target ratios of 12:1 and 6:1. After five hours incubation at 37°C, 100  $\mu$ L supernatant was counted in a gamma counter. Results were expressed as percent specific lysis and considered valid only if the ratio of spontaneous chromium release to maximum release was less than 30%.

**Lymphocyte proliferation assays (LPA).** LPA were performed at weeks -2, 0, 6, 14 and 26. PBMC were isolated and resuspended at a concentration of 1 x 10<sup>6</sup>/mL in RPMI 1640 media, supplemented with 10% heat inactivated human AB serum (Parramatta Blood Bank, NSW, Australia). Cells were incubated with 10  $\mu$ g/mL phytohaemagglutinin (Sigma, Taufkirchen, Germany), 0.1 Lf/mL Tetanus toxoid (CSL Ltd, Melbourne, Australia), 125 U/mL streptokinase (Lederle, Sydney, Australia), 10  $\mu$ g/mL gp160 (lot # 9248P) or 5  $\mu$ g/mL HIV-1 p55 (Quality Biologicals, Gaithersburg, MD, USA) at 37°C for seven days. Cells were pulsed with 1  $\mu$ Ci <sup>3</sup>H-thymidine (Amersham Biosciences Corp, Piscataway, NJ, USA) for 16 hours at 37°C, prior to harvesting. Incorporation of <sup>3</sup>H-thymidine was counted in Ecolite scintillation fluid (ICN Biomedicals, Sydney, Australia) on a beta counter.

**ELISpot assays.** ELISpot assays were performed at weeks -2, 0, 6, 14 and 26. Plates (96 well Multiscreen HA 0.45, Millipore Corporation, Billerica, MA, USA) were coated with 5- $\mu$ g/mL monoclonal antibody 1-DIK (Mabtech, Stockholm, Sweden) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed six times with 200  $\mu$ L PBS. An aliquot of 200  $\mu$ L 5% FBS in PBS was added to wells and plates were incubated for at least an hour at 37°C. Blocking media was discarded.

PBMC were isolated using the Ficoll Hypaque method. Effector cells (control and vaccinia-infected as CTL assays above), were dispensed at cell concentrations ranging from 2.5 x 10<sup>6</sup>-6.25 x 10<sup>6</sup>/well. PHA (10  $\mu$ g/ml) was added to the lowest dilution of uninfected cells as the positive cell

control and to three empty wells as the negative control. IFN- $\gamma$  (6 x 10<sup>4</sup> U/mL) was added to three empty wells as the positive reagent control and media alone to three wells as a negative control. Plates were incubated overnight at 37°C, 5% CO<sub>2</sub>.

On day 3, plates were washed three times with PBS containing 0.05% Tween-20, washed three times with PBS alone and incubated for 2.5 hrs at room temperature with 0.3  $\mu$ g/ml biotinylated monoclonal antibody 7-B6 (Mabtech, Stockholm, Sweden). The detection antibody was discarded. Plates were washed with 0.05% Tween in PBS, washed three times with PBS alone and incubated for 90 minutes at room temperature with 1 $\mu$ g/ml streptavidin-alkaline phosphatase (Sigma, Taufkirchen, Germany).

Immediately prior to the end of incubation substrate was prepared by dissolving one BCIP/NBT tablet (Sigma, Taufkirchen, Germany) in 10 ml water and filtering through a 0.45-micron filter. The conjugate was discarded. Plates were washed three times with 0.05% Tween-20 in PBS, washed three times with PBS alone and incubated with 100  $\mu$ L/well substrate at room temperature until development of dark spots. The reaction was stopped when PHA and IFN $\gamma$  wells were positive

(spots) and the blank wells were still white. The reaction was stopped by removing the plate backings and washing plates three times in tap water. Plates were air-dried and counted manually using a dissecting microscope and automatically with an image analysis system.

**Antibody responses.** Reactive anti-HIV antibody responses were assessed using a semi quantitative Western Blot (Diagnostic Biotechnology HIV BLOT version. 2.2, Genelabs Diagnostics, Singapore) at weeks 0, 6, 14 and 52. Reactive anti-FPV antibody responses were assessed using a semi-quantitative Western Blot assay in sera taken at weeks 0, 6, 14 and 52. Briefly, samples were separated by reducing SDS-PAGE and transferred via electrophoresis to a nitrocellulose sheet for staining by Western blot.

**Statistical analysis.** Safety data over 52 weeks and immunogenicity data over 26 weeks were subject to analysis. Immunogenicity data were analysed using the intention to treat principle in which all available data from all patients who received at least one vaccination were included. There were two formal comparisons between the randomised treatment groups in terms of safety and immunological endpoints. Firstly, the two active treatment arms PC and FC were combined and compared with the placebo group and secondly, the two active treatment arms were compared (FC versus PC).

Formal statistical comparisons of quantitative variables were performed using t-tests. Nonparametric tests were also performed yielding qualitatively identical results and are not presented. Binary variables were analysed using Fisher's exact or chi-square tests. All p-values and confidence intervals were two-tailed. There was no adjustment of p-values for multiple comparisons. All formal comparisons were performed without adjustment for baseline characteristics.

**Baseline characteristics.** Patient characteristics at baseline were summarized by randomized treatment group. Variables summarised included demographic data, ART history and HIV-related clinical parameters. There was no formal comparison of baseline characteristics across the three randomised treatment groups.

**Safety endpoints.** Analyses of safety endpoints included all patients who received any study medication, but were based on available data only. Changes from baseline (average of weeks -2 and 0) to each nominal study week were summarised by randomised treatment group for all safety endpoints. Formal statistical comparisons of safety data were limited to rates of adverse events and rates of detectable viral load. Rates of events were compared using Fisher's exact or chi-square tests.

**Immunological endpoints.** All analyses of immunological endpoints were on available data only (i.e., missing data were not imputed). Immunological

endpoints at baseline were summarised as the mean of measurements at weeks -2 and 0 and data collected at weeks -8 and 0 were summarised separately, with week 0 regarded as baseline.

The results of flow cytometry, T-cell proliferation, ELISpot and CTL assays were analysed as quantitative endpoints. T-cell proliferation, ELISpot and CTL data were also analysed as qualitative response variables.

For T-cell proliferation data, only the stimulation indexes were analysed. Patients with positive T-cell proliferation responses during follow-up were defined as those for whom the follow-up stimulation index was 5 or greater than their baseline value.

ELISpot results were regarded as true values if they were greater than 50 spot forming units per million cells and greater than twice the *vac.lac* control. If this was the case, the ELISpot value was taken to be the value minus twice the *vac.lac* control. Otherwise the result was taken to be zero. Patients with positive ELISpot responses at follow-up visits were defined as those with follow-up ELISpot values two or more times greater than their baseline value.

CTL results were regarded as true values if they were greater than the *vac.lac* control, in which case the *vac.lac* control value was subtracted. Otherwise, the CTL value was put to zero. This applied to all CTL results except for the positive control K562, for which the *vac.lac* values were not subtracted. A patient was regarded as having a positive CTL response if they had a follow-up value  $\geq 5\%$  above their baseline value. A second definition of response using a cut-off value of a 10% increase was also analysed at two effector/target ratios.

For each quantitative immunological result, the change from baseline (average of weeks -2 and 0) to each nominal study week was summarised by randomised treatment group. The proportions of patients with positive CTL responses at each nominal study week was also summarised by randomised treatment group. Nominal study weeks were calculated based on time windows and any patient with more than one value in a given time window had the average of their values taken.

Formal comparisons between randomised treatment groups for quantitative variables were based on two statistics for each endpoint, the change from baseline to week 14 and the time-weighted mean change from baseline over trial follow-up.

Randomised treatment groups were also compared formally in terms of the proportion of patients with positive CTL responses at week 14.

## RESULTS

**Patient baseline characteristics.** A total of 35 patients were randomised into the study (12, 11 and 12 patients to the placebo; partial and full construct vaccine arms respectively). One patient assigned placebo vaccine did not receive their third injection (due to international travel), but remained in follow-up and contributed to all analyses. All other patients received vaccinations as per protocol. Selected demographics and baseline clinical features are summarised in Table 2. For each variable the vaccine groups were balanced at baseline, with the exception of historical exposure to NNRTI and current use of NNRTI being more prevalent in PC recipients.

The cohort was exclusively male and predominantly caucasian. The average age of the cohort was representative of the broader HIV-infected community in Australia. Average CD4<sup>+</sup> cell counts were within normal ranges and plasma HIV viral load was uniformly below the limit of detection using an ultrasensitive assay. The average duration of ART use was 3.5 to 4 years; also indicating the average duration of HIV infection in the cohort.

Table 2 Immunogenicity data

Assay System	Placebo	PC	FC
<b>ELISpot</b>			
Sfu/10 <sup>6</sup> PBMC—mean baseline $\pm$ SD	15 $\pm$ 47	21 $\pm$ 42	11 $\pm$ 21
Sfu/10 <sup>6</sup> PBMC—time weighted mean change $\pm$ SD	64 $\pm$ 137	-5 $\pm$ 16	19 $\pm$ 30
Percentage patients with positive response (any study week)	33.3	0	50
<b>Lymphoproliferation</b>			
Stimulation index -time-weighted mean change $\pm$ SD	-3.5 $\pm$ 16.5	3.3 $\pm$ 11.4	-1.2 $\pm$ 9.5
Percentage patients with positive response (any study week)	33.3	72.7	41.7
<b>Chromium release</b>			
Number of patients (%) with positive response	0	1 (9)	1 (8)

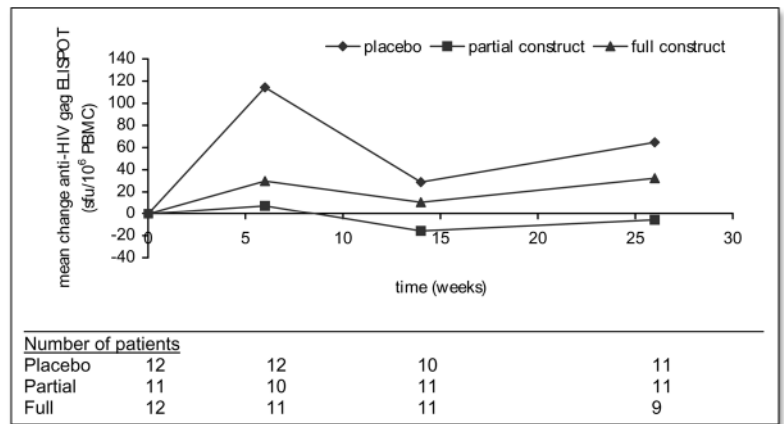


Figure 1. Mean change from baseline anti-HIV gag responses assessed by ELISpot assays.

**Safety data.** There were three serious adverse events reported in three patients during the study. A placebo recipient developed severe agranulocytosis secondary to management of recurrent hypothyroidism and unrelated to the study vaccines approximately 11 months after first vaccination. A PC recipient presented 6.5 months after first vaccination with clinical symptoms and signs including lethargy and fatigue. This individual had significant elevations in serum liver transaminases, alkaline phosphatase and bilirubin. Further evaluation confirmed acute viral hepatitis A infection that resolved 3–6 months after initial presentation. An additional PC recipient was found to have significantly elevated serum liver transaminases at week 0, prior to first vaccination, which resolved approximately one month later.

The only adverse event that occurred in at least 10% of patients at least once with at least possible relatedness to vaccination was injection site reactions (ten patients—two placebo, three partial construct and five full construct recipients). There was no evidence that the frequency or severity of laboratory adverse events was associated with either of the randomly assigned vaccines in this study.

**T-cell subset enumeration.** There were no differences between randomly assigned treatment groups for mean change from baseline CD4<sup>+</sup> cell count changes across the 52 weeks of study (data not shown). Furthermore, there were no observable differences between treatment groups for a range of T-cell subset populations expressed as either absolute cell numbers or percentage of total T-cell numbers (data not shown).

**Plasma HIV viral load and changes to antiretroviral therapy.** Eight episodes of detectable HIV viremia (defined as any plasma HIV RNA > 50 copies/mL plasma) were experienced in five patients across the 52 weeks of study, with no difference between vaccine groups.

Five changes to baseline ART were noted across the duration of the study (placebo = 1, PC = 2, FC = 2), due to pill burden and toxicity.

Table 3 **Anti-FPV Antibody Responses**

Study Week	Placebo	PC	FC
Baseline	1 (8.3)	0	0
6	0	8 (73)	10 (83)
14	0	9 (82)	11 (100)
52	0	7 (70)	9 (75)

Reactive anti-FPV antibody responses, as assessed using semi-quantitative Western blot are presented as number of patients nonreactive to reactive (% of evaluable group).

#### Immunogenicity assays

**ELISpot responses.** Study ELISpot data are summarised in Table 2. Baseline anti-HIV ELISpot responses to HIV gag were low in all three vaccine groups. Following vaccination there was little if any change in anti HIV gag responses measured by ELISpot assay (Fig. 1). There were no significant differences in time-weighted mean change from baseline anti-HIV gag immune responses for the combined recipients of the PC and FC versus placebo recipients (mean difference = -56 sfu/10<sup>6</sup> PBMC (95% CI—115, 3.0;  $p = 0.062$ ). There was a significant increase in anti -HIV gag responses for FC recipients, compared with PC recipients (mean difference = 24 sfu/10<sup>6</sup> PBMC 95% CI—3, 46;  $p = 0.026$ ). Results of ELISpot assays using HIV pol antigens did not reveal any significant differences between any treatment group (data not shown).

There was no significant difference between the proportions of combined PC and FC recipients with placebo recipients who had positive anti-HIV gag ELISpot responses ( $p = 0.955$ ). However, the proportion of patients with positive anti-HIV gag responses was significantly greater in those receiving FC, from those receiving PC ( $p = 0.024$ ).

**<sup>51</sup>Cr release cytotoxic lymphocyte responses.** The proportion of patients with any specific lysis > 5 x baseline at any time point during the study is summarised in Table 1. There was no apparent difference between vaccine groups for this endpoint. At an E:T ratio of 12:1, for the comparison of time weighted mean change from baseline in anti-HIV gag responses there was a nonsignificant treatment effect estimate for the combined recipients of FC and PC versus placebo of 0.48 (95% CI—3.18, 2.23;  $p = 0.723$ ). The comparison of responses arising in recipients of FC versus PC revealed a treatment effect estimate of 0.24 (95% CI—0.49, 0.96;  $p = 0.507$ ).

**Lymphoproliferative responses.** Lymphoproliferative responses to stimulation by HIV-1 p55 antigen (Table 2) revealed no significant differences in time-weighted mean change in anti-HIV gag lymphoproliferative responses for the combined recipients of the PC and FC versus placebo recipients (mean difference = 4.4 SI 95% CI—4.8, 13.7;  $p = 0.337$ ). Similarly, there were no statistically significant differences in anti -HIV gag lymphoproliferative responses (mean difference = -4.5 SI 95% CI—13.6, 4.6;  $p = 0.314$ ), on comparison of FC versus PC.

Furthermore, there were no statistically significant differences, when comparing the proportions of combined PC and FC recipients with placebo recipients who had positive anti-HIV gag lymphoproliferative responses ( $p = 0.343$ ) or when comparing the proportion of patients receiving FC with those receiving PC that had positive anti-HIV gag responses ( $p = 0.280$ ).

**Anti-HIV antibody responses.** No patient in the placebo or any randomly assigned vaccine group generated anti-HIV p55 antibody responses at levels higher than those present at baseline over the period of study (data not shown).

**Anti-FPV antibody responses.** Only one patient (PC recipient) had reactive anti-FPV antibodies at baseline. After two vaccinations nearly all vaccine recipients had detectable anti-FPV antibodies (Table 3). Responses in the majority of vaccine recipients persisted for periods up to 40 weeks following last vaccination.

## DISCUSSION

This clinical study examined the safety and immunogenicity of two candidate fowlpox vaccine constructs in HIV-infected volunteers who had maintained good virologic control following initiation of ART during or shortly after primary HIV infection.

Use of all classes of available ART was similar both historically and at enrolment across the three study arms. A small imbalance in historical use of NNRTI drugs was noted in addition to imbalances in baseline use of various classes of antiretroviral drugs. The significance of this finding is not known, although recent studies have demonstrated that NRTI-containing and PI-containing regimes have similar effects on immune reconstitution.<sup>30</sup> Hence, it is reasonable to assume that the imbalances in type of ART were irrelevant in the current study.

Like the more widely investigated recombinant canary pox vaccines,<sup>31</sup> the current rFPV vaccines exhibited good safety profiles with no difference from placebo in terms of frequency or severity for reported adverse events. Only mild localised injection site reactions were consistently associated with active vaccination in a substantial number of participants.

Samples derived from patients in this study were interrogated for T-cell immune responses at time points following vaccination that were selected to provide the best chance of disclosing immunogenicity. Consistently, in each of three laboratory reporting systems employed in this protocol, the demonstrated levels of HIV-specific T-cell immune responses were no higher than those observed contemporaneously in individuals who received placebo vaccines.

Neither the PC (fowl pox expressing HIV *gag/pol*) nor the FC (fowlpox expressing HIV *gag/pol* and human IFN- $\gamma$ ) candidate vaccines are T-cell immunogens when administered to individuals with HIV infection, as described by the immunogenicity assessments performed in this protocol. Analysis of data included qualitative and quantitative assessments. It is noteworthy that irrespective of the formal comparisons, the observed levels of ELISpot positive responses were marginal relative to published literature for other candidate vaccines for HIV.<sup>32</sup>

One noteworthy observation was the statistically significant increase in ELISpot anti-HIV gag responses for recipients of the full construct relative to recipients of the partial construct. This modest mean difference of 24 sfu/10<sup>6</sup> PBMC ( $p = 0.026$ ) must be viewed cautiously for a number of reasons. Firstly, the same statistic and analysis for the comparison of the combined vaccine recipients versus placebo was not statistically significant ( $p = 0.062$ ). Secondly, this analysis has generated very large numbers of  $p$ -values and the likelihood of encountering a false significant value in this setting is high.

These data are perhaps surprising in terms of the available literature on candidate HIV vaccines, especially that of the closely related family of canary pox vectors. A series of recombinant canary pox HIV-1 vaccines (predominately derived from the ALVAC strain) have been shown to possess modest T-cell immunogenicity in HIV-infected<sup>30</sup> and HIV-uninfected subjects.<sup>33-36</sup> In one of these studies, HIV-specific CD8<sup>+</sup> cytotoxic T cells were detected at least once in 33% of seronegative volunteers receiving vCP205, either alone or in combination with recombinant gp120.<sup>35</sup> Neutralizing antibody responses were elicited in 94% of volunteers receiving VCP205 in combination with rgp120 and gag-specific CD4<sup>+</sup> T helper cells were also elicited. The HIV-specific T cell immune responses observed can be variable and transient in nature.<sup>37</sup> Nonetheless, the absence of such responses in the current study is robust across a range of

assay systems. Unit doses of fowl pox virus (pfu/mL) and canary pox virus (TCID<sub>50</sub>/mL) can be regarded as largely equivalent. As such our study involved the administration of marginally higher unit doses of vaccine than previously reported trials with canary-pox.<sup>35,38</sup> The biological assays used to establish the virus titre are notoriously variable and we do not know what method was employed to describe the specific activity of the ALVAC studies. We suspect that vaccine dose does play a role in the induction of T-cell mediated immune responses and would plan to use higher doses in any subsequent trials of the vaccine. It is noteworthy that considerable higher doses<sup>39</sup> (i.e.,  $1.5 \times 10^9$  pfu) have been used when an alternative strain of fowlpox virus has been utilised in cancer vaccine applications.

Recently, data have been presented that describe T-cell immune responses in HIV-infected individuals treated with antiretrovirals at the time of primary HIV infection and then ALVAC vaccine and boosted with a recombinant purified protein.<sup>32,38</sup> In a more recent study, patients receiving combination antiretroviral therapy received modified vaccinia virus Ankara expressing HIV-1 *nef* followed by cessation of antiretroviral therapy. New CD4<sup>+</sup> and CD8<sup>+</sup> anti-HIV responses were observed in most patients and subsequent to cessation of combination antiretroviral therapy these were associated with modest changes in the recrudescence of virus replication.<sup>40</sup> In a larger randomized study, recipients of a canarypox virus vector vaccine (ALVAC-vCP1433) in combination with a lipopeptide adjuvant (Lipo-6T) and cyclical recombinant interleukin-2 (IL-2) developed multi-epitope CD4<sup>+</sup> HIV-specific immune responses more frequently than unvaccinated controls.<sup>41</sup> Vaccinated subjects in this study also appeared to have a more favorable profile of plasma HIV recrudescence after cessation of combination antiretroviral therapy compared with unvaccinated controls. More recently, promising results have been presented following immunisation of HIV-1 infected patients with autologous dendritic cells treated *ex vivo* with inactivated HIV-1.<sup>41</sup> In this study following immunization, reductions in plasma HIV RNA levels were observed and these were correlated with sustained CD4<sup>+</sup> and CD8<sup>+</sup> T cell anti-HIV immune responses. It is likely that induction of effective anti-HIV cell mediated immune responses will require effective stimulation of professional antigen presenting cells such as dendritic cells.

There is some evidence to indicate that vaccine recipients generated reactive anti-FPV antibodies soon after administration of candidate vaccines. The evolution of reactive anti-FPV antibodies was manifest by week 6 in nearly all recipients of candidate vaccines in this study. Detectable anti-FPV antibodies remained for nearly one year after most recent exposure to any candidate FPV vaccine. Whether or not these antibodies had the capacity to neutralize further administration of FPV candidate vaccines is not known. It is possible that the evolution of antibodies may have played some role in preventing the generation of T-cell mediated immune responses. This hypothesis would require that detectable antibodies neutralised administered fowlpox. However, studies of fowlpox vector vaccines in oncology have shown that reactive fowlpox antibodies do not prevent the generation of significant T cell immune responses to fowlpox-encoded antigens (Panacali D, personal communication).

Despite evidence of safety neither of the candidate vaccine constructs evaluated in this protocol appear to possess any T-cell mediated anti-HIV immunogenic properties.

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