

# Mucosally-administered human–simian immunodeficiency virus DNA and fowlpoxvirus-based recombinant vaccines reduce acute phase viral replication in macaques following vaginal challenge with CCR5-tropic SHIV<sub>SF162P3</sub>

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## Abstract

Further advances are required in understanding protection from AIDS by T cell immunity across mucosal sites of virus transmission. We analysed a set of multigenic HIV and SHIV DNA and Fowlpoxvirus (FPV) prime and boost vaccines for immunogenicity and protective efficacy in outbred pigtail macaques when delivered via mucosal surfaces (intranasally or intrarectally). Intranasally delivered DNA, even when adjuvanted and given as a fine droplet spray, was neither immunogenic nor protective in macaques. Some protection from acute infection with a pathogenic vaginal SHIV<sub>SF162P3</sub> challenge was, however, observed with a regimen involving intramuscular DNA vaccine priming followed by either intranasally or intrarectally delivered rFPV boosting. Interestingly, animals boosted with rFPV vaccine via either of these mucosal routes had poor circulating T cell responses prior to challenge with SHIV compared to those boosted via the intramuscular route. Nevertheless, the mucosally-vaccinated animals generated equivalent anamnestic mucosal and systemic SHIV-specific CD4 and CD8 T cell responses following SHIV administration, with significant reduction in acute plasma viremia against this vaginal challenge. Our data suggest strategies for effective priming of partial immunity to mucosal HIV-1 exposure utilizing systemic prime and mucosal boost vaccination strategies.

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## 1. Introduction

Transmission of HIV-1 occurs predominantly across genital or rectal mucosa, most commonly by heterosexual intercourse. HIV-1 dissemination after mucosal infection involves stepwise spread from mucosal draining lymph nodes to distant systemic lymphoid tissues. Proposed dissemination routes include migration of infected antigen-presenting cell populations and early infection of resident resting CD4<sup>+</sup> T

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cell populations [1–5]. Selective recruitment and retention of CCR5+ CD4<sup>+</sup> T cells also occurs in the genital tract and this may aid transmission of CCR5-tropic HIV-1 strains [6].

The ability of virus-specific immune responses to prevent or limit viral replication in genital or rectal mucosal tissues is likely to be critical for defense against HIV-1 infection. An important goal for vaccination is, therefore, to develop strategies that will induce durable immune responses both at mucosal sites of transmission as well as in the systemic circulation. Mucosal immune responses are often regulated independently of systemic immunity. Lymphocyte homing to mucosal tissues is mediated partly by cell surface integrins interacting with their ligands on local blood vessel walls [7]. The genital tract lacks organized lymphoid aggregates, although it is replete with potent antigen-presenting cells [2], raising the possibility that induction of genital responses may occur elsewhere, including other mucosal sites, followed by re-circulation of immune cells into these tissues. Clearly, a greater understanding of mechanisms underlying the generation of immune responses in the genital tract will be critical for the rational development of protective vaccination strategies. Nevertheless, evaluation of novel mucosal delivery strategies of promising HIV/SIV vaccine strategies is clearly warranted.

A vaccine that prevents infection of any host cells will need to induce neutralizing antibodies (NAb) at the mucosal site of infection. Instillation of the human monoclonal NAb b12 into the vagina protected 9 of 12 macaques from subsequent vaginal challenge with SHIV<sub>SF162P4</sub> [8]. However, in the absence of reliable vaccine strategies to induce broad NAb, a strong antiviral T cell response at mucosal sites could restrict viral replication and provide partial immunity. An enrichment of HIV-specific CTL in the cervix relative to blood is observed in high exposure, persistently sero-negative sex-worker cohorts [9]. Furthermore, Gag-specific, tetramer-positive T cell frequencies of up to 14% of the CD8<sup>+</sup> T cell population were found in vaginal and cervical lamina propriae of macaques infected with SIV or SHIV [10]. Unfortunately, few immunization strategies specifically direct T cell-mediated immune responses to these tissues. Purely systemic vaccination strategies (e.g. IM), while potentially inducing some mucosal T cell immunity [11], typically do not induce T cells with mucosal homing markers and are therefore unlikely to induce immunity at mucosal sites as effectively as alternative mucosal or live attenuated vaccination strategies [7]. While having limitations as a universal vaccine approach, the local delivery of peptide immunogens has generated solid mucosal CD8<sup>+</sup> T cell responses in both mice and non-human primates [12,13]. However, mucosal delivery of attenuated poxvirus vectors, such as canarypoxviruses, has generally induced poor mucosal T cell immunity without prior priming in humans [14]. Other vector-based approaches, using constructs designed to replicate in mucosal tissues, have shown greater promise for induction of mucosal immunity to SIV/HIV-1 [15–17], although strategies restricted to the use of a single virally-vectored vaccine may also have limited potential due

to weaker immunogenicity than prime-boost regimens and the development of anti-vector immunity in the host.

Systemically-delivered heterologous prime and boost HIV vaccine strategies involving priming by DNA vaccination and boosting with recombinant attenuated poxvirus vectors encoding common HIV or SIV antigens reliably induce high levels of circulating T cell immunity in outbred non-human primates [18–22]. Avian poxviruses such as fowlpoxvirus (FPV) have potential advantages over vaccinia virus-based approaches since anti-vaccinia responses from prior smallpox vaccination limits the widespread utility of recombinant vaccinia virus vaccines. DNA/poxvirus vaccine-induced SHIV-specific T cell responses correlate with partial protection from virulent SHIV infection [22,23]. When given in prime-boost combination via mucosal routes, similar DNA and poxvirus-based vaccines have been shown to induce mucosal T cell immunity in both mice and non-human primates [24–27] (Ranasinghe et al., unpublished data). Administration of DNA and recombinant Modified Vaccinia Ankara vaccines expressing SHIV antigens via mucosal routes show promise against CXCR4-utilizing SHIV<sub>89,6P</sub> challenge in non-human primates [26,27]. CXCR4-utilizing SHIV strains target different CD4 T cells than CCR5-utilizing viruses, which are most commonly transmitted via the heterosexual route and are generally more difficult to neutralize and protect against [21,28]. Furthermore, our recent studies in mice indicate that delivery of DNA and poxvirus HIV vaccines in mucosal–mucosal (intranasal) or systemic–mucosal combinations is also immunogenic for both systemic and mucosal T cell responses, notably in the lymph nodes draining the genital and rectal mucosa (Ranasinghe et al., unpublished data, [24]). We therefore evaluated the immunogenicity and protective efficacy of mucosally-delivered DNA and rFPV vaccines against a virulent vaginal CCR5-utilizing SHIV challenge in non-human primates.

## 2. Methods

### 2.1. Monkeys

Juvenile *Macaca nemestrina* were free from HIV-1/SIV/simian retrovirus (SRV) infection, housed under physical containment level 3 (PC3) conditions and anaesthetised with ketamine (10 mg/kg intramuscular (IM)) prior to procedures. All experiments were performed according to National Institutes of Health guidelines on the care and use of laboratory animals, and were approved by the University of Melbourne and CSIRO Livestock Industries Animal Experimentation and Ethics Committees.

### 2.2. DNA vaccinations

The multigenic DNA vaccines, pHIS-HIV-B and pHIS-SHIV-B, have been previously described [22,29]. Briefly, the pHIS-SHIV-B encoded full length unmutated SIV<sub>mac239</sub>

Table 1  
Mucosal HIV vaccine regimens

Group	N	DNA priming vaccine				rFPV boosting vaccine			
		Construct <sup>a</sup>	Route	Dose (mg)	Timing (week)	Construct <sup>a</sup>	Route	Dose (pfu)	Timing (week)
Control	4	pHIS	IN, IM	1	0, 3	FPV-M3	IM	$5 \times 10^7$	6, 10
IM/IM	6	pHIS-HIV-B	IM	1	0, 3	rFPV-HIV-B	IM	$5 \times 10^7$	6, 10
IN/IM	6	pHIS-HIV-B	IN	1	0, 3	rFPV-HIV-B	IM	$5 \times 10^7$	6, 10
IN/IN	6	pHIS-HIV-B	IN	1	0, 3	rFPV-HIV-B	IN	$5 \times 10^7$	6, 10

IN, intranasal; IM, intramuscular; pfu, plaque forming units.

<sup>a</sup> All constructs encode HIV-1 subtype B antigens.

Gag and Pol, HIV-1<sub>NL4.3</sub> Tat, Rev and Vpu and the 5' one third of HIV-1<sub>AD8</sub> Env. The pHIS-HIV-B encoded a mutated HIV-1<sub>NL4.3</sub> Gag and Pol (minus integrase), HIV-1<sub>AD8</sub>, Tat, Rev and Vpu and the 5' one third of HIV-1<sub>AD8</sub> Env. Genes were inserted into vector pHIS-64 (Coley Pharmaceutical Group, Wellesley MA) behind the human cytomegalovirus immediate-early promoter. Plasmid vector pHIS-64 has kanamycin resistance, bovine growth hormone polyA termination signal, and 64 primate-optimised CpG motifs in addition to those that are naturally present. Empty vector plasmid DNA, pHIS, served as a control vaccine. Plasmid DNA in normal saline was injected at 1 mg in 1 mL IM. For intranasal administration in the HIV-1 trial the pHIS-HIV-B at 8 mg/mL was complexed with Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) and 1 mg of DNA in 0.37 mg Lipofectamine applied atraumatically to the nasal mucosa with a pipette. For the SHIV trial, the pSHIV-HIV-B plasmid at 7.5 mg/mL was combined with DOTAP liposomal transfection reagent (Roche, Indianapolis, IN) and 1 mg of DNA in 0.27 mg of DOTAP instilled intranasally using a fine droplet nasal spray pump (Valois of America, Congers, NY), (Tables 1 and 2).

### 2.3. Recombinant fowlpoxvirus vaccines

Construction of the multigenic rFPV vaccines rFPV-HIV-B, encoding mutated HIV-1<sub>NL4.3</sub> Gag and Pol (minus

integrase) for the HIV-1 study and rFPV-SIVGag/Pol and rFPV-HIV-AE(Env) for the SHIV study have been described [22,29,30]. Briefly, rFPV expressing either SIV<sub>mac239</sub>Gag/Pol or mutated HIV-1<sub>NL4.3</sub> Gag/Pol, were constructed by inserting the promoter-*gag/pol* PCR amplicon into pKG10a for insertion into FPV-M3 at the F6,7,9 site. PCR primers used the FPV Early/Late promoter and an early transcription terminator for insertion into fowlpoxvirus vector FPV-M3. HIV-1<sub>93TH254</sub> *env* (mutated to remove the middle third containing the CD4 binding region) was similarly amplified with FPV promoter and terminator sequences and inserted into the plasmid vector pCH34 for construction of a recombinant FPV with HIV Env expressed from the REV insertion site. Recombinants were selected on the basis of co-expression of the *Escherichia coli* gpt gene and plaque purified on the basis of co-expression of the *E. coli* beta-galactosidase gene. rFPV vaccines were prepared in saline and  $5 \times 10^7$  pfu in 1 mL injected once into the gluteal muscle or, after a 30 min digestion with trypsin (Sigma; 1 mg/mL) instilled atraumatically intranasally or intrarectally twice over 2 days (100  $\mu$ l of a  $5 \times 10^7$  pfu stock) with a pipette (Tables 1 and 2).

### 2.4. IFN $\gamma$ ELISpot assay

Enumeration of antigen-specific IFN $\gamma$ -secreting cells was assessed using a Monkey IFN $\gamma$  ELISpot commercial kit (U-

Table 2  
Mucosal SHIV vaccine regimens

Group	N	DNA priming vaccine				rFPV boosting vaccine			
		Construct	Route	Dose (mg)	Timing (week)	Construct	Route	Total dose (pfu)	Timing (week)
Control	4	pHIS	IN, IM	1	0, 4, 8	FPV-M3	IM IN <sup>a</sup> IR <sup>a</sup>	$5 \times 10^7$	13, 17
IM/IM	4	pHIS-SHIV-B	IM	1	0, 4, 8	rFPV-SIVGag/Pol rFPV-HIV-AE(Env)	IM	$5 \times 10^7$	13, 17
IM/IN	4	pHIS-SHIV-B	IM	1	0, 4, 8	rFPV-SIVGag/Pol rFPV-HIV-AE(Env)	IN <sup>a</sup>	$1 \times 10^8$	13, 17
IM/IR	4	pHIS-SHIV-B	IM	1	0, 4, 8	rFPV-SIVGag/Pol rFPV-HIV-AE(Env)	IR <sup>a</sup>	$1 \times 10^8$	13, 17
IN/IN	4	pHIS-SHIV-B	IN	1	0, 4, 8	rFPV-SIVGag/Pol rFPV-HIV-AE(Env)	IN <sup>a</sup>	$1 \times 10^8$	13, 17

IN, intranasal; IM, intramuscular; IR, intrarectal; pfu, plaque forming units.

<sup>a</sup> rFPV vaccines were delivered to the mucosal sites over two consecutive days:  $5 \times 10^7$  pfu each day.

CyTech, Utrecht, The Netherlands) as previously described [31]. Briefly, freshly isolated PBMC were stimulated with Aldrithiol-2-inactivated whole HIV-1<sub>MN</sub> or SIV<sub>mne</sub> or control microvesicles (5 µg/mL, kindly provided by Dr. Jeff Lifson, AIDS Vaccine Program, National Cancer Institute, MD) or with HIV-1<sub>HXB2</sub> Gag (3 µg/mL) or SIV<sub>mac239</sub> Gag and Pol overlapping 15mer peptide pools at 1 µg/mL/peptide (kindly supplied by the NIH AIDS Research and Reference Reagent Program) for 18 h. Cells were washed twice, transferred to anti-IFN $\gamma$  monoclonal antibody-coated transparent 96-well plates containing antigen and incubated for a further 5 h to capture IFN $\gamma$  production. Cells were lysed and plates washed prior to 1 h incubation with biotinylated anti-IFN $\gamma$  polyclonal rabbit antibody, followed by 1 h incubation with gold-labelled anti-biotin IgG antibody. IFN $\gamma$  spots were developed and analyzed by an automated ELISpot plate reader (AID, Strassberg, Germany). Results were normalized to antigen-specific IFN $\gamma$ -secreting precursor frequency per 10<sup>6</sup> PBMC. For ELISpots performed on mucosal T cell populations, the cells were rested for 24 h by culture in RPMI plus 5% foetal calf serum prior to use in the above assay.

### 2.5. Intracellular IFN $\gamma$ staining (ICS)

Induction of antigen-specific intracellular IFN $\gamma$  expression in CD3<sup>+</sup>CD8<sup>+</sup> or CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes was assessed by flow cytometry as previously described [31,32]. Briefly, 200 µl whole blood was incubated with 1 µg/mL overlapping 15mer peptide pools dissolved in DMSO or DMSO alone (control) and the co-stimulatory antibodies CD28 and CD49d (BD Biosciences Pharmingen San Diego CA) for 7 h. Brefeldin A (10 µg/mL; Sigma) was included during the last 5 h of the incubation. Anti-CD3-PE, anti-CD4-FITC and anti-CD8-PerCP (BD) antibodies were added to each well and incubated for 30 min. RBC were lysed (FACS lysing solution, BD), washed with PBS and the remaining cells permeabilized (FACS Permeabilizing Solution 2, BD). Permeabilized cells were then incubated with anti-human IFN $\gamma$ -APC antibody (BD) prior to fixing with formaldehyde and acquisition (FACSscan, BD). Acquisition data was analyzed using CellQuest (BD). The percentage of antigen-specific gated lymphocytes expressing IFN $\gamma$  was assessed in both CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> lymphocyte subsets.

### 2.6. Lymphoproliferative responses

Lymphoproliferative responses were assessed by standard <sup>3</sup>H-thymidine incorporation assay to 1 µg/mL SIV Gag peptide pool, 10 µg/mL inactivated SIV or control antigens as described [18] and also phenotyped by CFSE proliferation studies [22]. PBMC were stained with the fluorescent dye CFSE (5 µM; Molecular Probes, Eugene OR) prior to culturing for 6 days with the SIV gag peptide pool or inactivated SIV as above. Cells were washed in PBS and phenotyped using monoclonal antibodies: PE-conjugated anti-human CD3 (clone SP34; BD) PerCP-conjugated anti-human CD4

(clone L200; BD) and APC-conjugated anti-human CD8 (clone Leu-2a; BD). Cells were formaldehyde-fixed before FACS analysis. Proliferating CD4 or CD8 T cells were expressed as the percentage of CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> cells emitting CFSE of a lower intensity than the CFSE intensity of the non-dividing T-lymphocyte subset.

### 2.7. SHIV<sub>SF162P3</sub> challenge of macaques

To assess vaccine efficacy, all 24 female macaques were inoculated atraumatically intravaginally with SHIV<sub>SF162P3</sub> (3 × 10<sup>3</sup> TCID<sub>50</sub>/mL) in 0.5 mL doses over 2 days (total 6 × 10<sup>3</sup> TCID<sub>50</sub>/mL). SHIV<sub>SF162P3</sub> was obtained from the NIH AIDS Research and Reference Reagent Program (contributed by Dr. Janet Harouse), and expanded on CD8 T cell-depleted human PBMC prior to use [33–35]. SHIV viremia was quantified by reverse-transcriptase real-time PCR and depletion of peripheral CD4 T cells monitored by flow cytometry as previously described [31].

### 2.8. Mucosal T cell collection

To isolate T cells from endocervical and rectal mucosal tissues and their draining lymph nodes, 5–10 1 mm<sup>3</sup> pieces of tissue samples were digested with a 1–2 mL solution of collagenase (2 mg/mL, Calbiochem, USA), dispase (2.4 mg/mL, Calbiochem, USA), and DNase I (125 µg/mL, Calbiochem, USA) in RPMI containing 10% FCS and incubated at 37 °C for 60 min with regular vortexing. The cells were then filtered through sterile gauze to remove debris, washed in RPMI, passed through a sterile strainer (Falcon 100 µm Nylon) and washed again. RBC were then removed by incubating the cell preparation at 37 °C for 5 min with a 0.15 M NH<sub>4</sub>Cl red cell lysis buffer, and the cell preparation then washed twice and counted.

### 2.9. TZM-bl luciferase reporter gene assay for neutralizing antibodies

Neutralization was measured as a function of reductions in luciferase reporter gene expression after a single round of virus infection in TZM-bl cells as described [36]. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. These cells are engineered to express CD4 and CCR5 [37] and contain integrated reporter genes for firefly luciferase and *E. coli*  $\beta$ -galactosidase under control of an HIV-1 LTR [38]. Briefly, cell free SHIV<sub>SF162P3</sub> (200 TCID<sub>50</sub>, amplified on human PBMC) was incubated with serial dilutions of test samples in triplicate in a total volume of 150 µl for 1 h at 37 °C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 µl of growth medium containing 75 µg/mL DEAE dextran and 2.5 µM indinavir) were added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After a 48 h incubation, 100 µl of cells



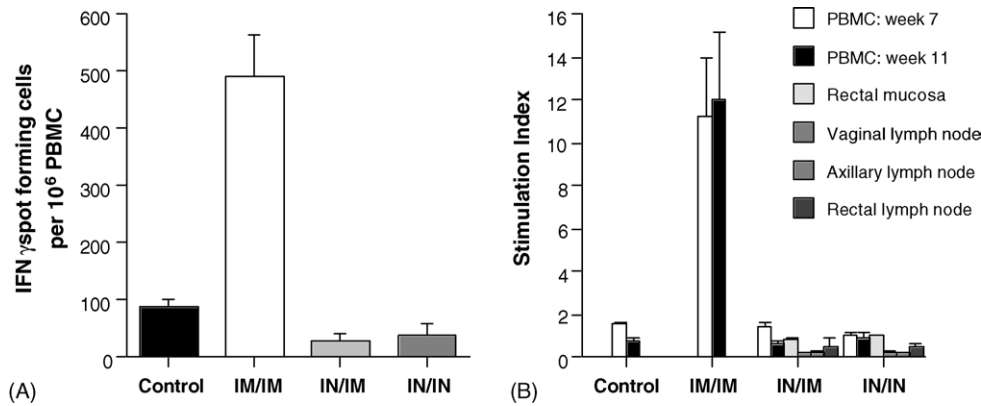


Fig. 1. T cell immunity following HIV-1 DNA/rFPV prime/boost vaccine regimens delivered intranasally. (A) HIV-1 Gag overlapping peptide-specific T cell responses by IFN $\gamma$  ELISpot after the rFPV boost in the DNA/rFPV vaccine regimens as noted in Table 1. Mean (+standard error) responses are shown for each group. (B) T cell proliferative responses by <sup>3</sup>H-thymidine incorporation method to HIV Gag overlapping peptides across the vaccine groups in blood and various tissues. PBMC HIV-specific proliferative responses were assessed after both the first (week 7) and second (week 11) rFPV boost. Rectal mucosal and lymph node HIV-specific T cell proliferation in the groups receiving IN vaccines was assessed at autopsy at week 12.

were transferred to a 96-well black solid plates (Costar) for measurements of luminescence using Bright Glo substrate solution as described by the supplier (Promega). Neutralization titers represent the dilution at which relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLUs.

### 2.10. Statistical considerations

The primary endpoint for the SHIV challenge study, established prior to starting the study, were differences in outcome of SHIV challenge between groups as defined by a time-weighted area-under-the-curve for viral RNA copy number (viral load) over the first 4 weeks of acute SHIV infection. This acute infection primary endpoint was chosen since acute infection is relatively uniform in naïve macaques and predicts long term outcome. Based on previous studies with this challenge virus in rhesus macaques [33–35,39], we calculated that 4 macaques/treatment group powered the study (80%,  $2\alpha = 0.05$ ) to detect 1.09 log<sub>10</sub> differences in four vaccinated versus eight control macaques. Statistical comparisons of secondary endpoints of peak SHIV viral load and immunogenicity between vaccine groups utilised pair-wise comparisons without compensation for multiple analyses.

## 3. Results

### 3.1. HIV-1 macaque study

In the first set of experiments, we compared the immunogenicity of HIV-1-encoding DNA and rFPV vaccines delivered intranasally (IN), systemically (IM) or in mucosal and systemic combination (IN/IM) in groups of four to six pig-tail macaques (Table 1). High levels of T cell immunity in peripheral blood were generated when DNA and rFPV vaccines were administered by the IM route (IM/IM), as

we have previously reported [18,22,29]. The two groups of animals primed with DNA vaccines by the IN route and boosted with rFPV, either by the IN route (IN/IN) or the IM route (IN/IM) failed to generate significant HIV-specific T cell immunity in peripheral blood as demonstrated by IFN $\gamma$  ELISpot assays (Fig. 1A). A similar pattern of responses was observed by HIV-specific T cell proliferation, with proliferation in response to HIV-1 antigens observed only in PBMC obtained from the IM/IM group after either 1 or 2 rFPV boosts (Fig. 1B). In addition, no HIV-1 antibodies were detected by EIA or western blot in plasma from macaques immunized with the IN/IN or IN/IM regimens (data not shown). In contrast to animals given rFPV IM [29], no anti-FPV antibodies were detected by western blot in macaques given IN rFPV (data not shown), suggesting limited presentation of rFPV antigens to the circulating immune system following delivery by this mucosal route.

To examine mucosal T cell immunity in the mucosally (IN)-immunized animals, we obtained rectal mucosal tissues and lymph nodes draining the rectum and genital tract at autopsy and performed both IFN $\gamma$  ELISpot assays and lymphoproliferation assays for HIV-specific immunity. Unfortunately, the IFN $\gamma$  ELISpot assays on rectal mucosal and genital lymph node mononuclear cell preparations were complicated by high spontaneous levels of IFN $\gamma$  release and the results were inconclusive (data not shown). The lymphoproliferation assays, however, demonstrated no HIV-specific mucosal T cell proliferative activity in the IN/IN or IN/IM immunised macaques (Fig. 1B).

### 3.2. Immunogenicity of mucosal SHIV vaccines in macaques

The absence of demonstrable T cell responses in the IN/IN and IN/IM vaccine groups in the HIV-1 study suggested a failure of immune priming following IN instillation of the DNA vaccine, since a boost in these responses was observed fol-

lowing IM rFPV administration in the IM/IM group but not in the IN/IM group (Fig. 1). In our second set of experiments, a SHIV-based study, we addressed this issue by delivering DOTAP lipid-complexed DNA vaccine three times to the nasal epithelium using a fine droplet sprayer (IN/IN group), a strategy that had proven immunogenic in our murine studies ([24] Ranasinghe et al., unpublished data). In parallel experiments, we also studied the efficacy of IN and IR delivery of rFPV for boosting mucosal responses in systemically (IM) DNA-primed animals based on the success of these approaches in mice (IM/IN and IM/IR groups). We evaluated both the immunogenicity of these vaccine regimens and their capacity to ameliorate a subsequent pathogenic mucosal (vaginal) SHIV challenge. Five groups of pigtail macaques were primed with SHIV-expressing DNA (three doses) and boosted with rFPV expressing the shared SIV<sub>mac239</sub> Gag and Pol proteins (two doses) in the combinations shown in Table 2.

The kinetics, magnitude and phenotype of the systemic (circulating) cellular immune responses induced by these vaccines were quantified by antigen-specific T cell production of IFN $\gamma$  using both ELISpot and ICS assays (Fig. 2). As expected, the IM/IM DNA/rFPV vaccine regimen was the most immunogenic, inducing a mean peak response to whole inactivated SIV of 811 SFC/10<sup>6</sup> PBMC (range 595–1158), demonstrating vigorous recognition of processed viral particles (Fig. 2A). The peak mean response to SIV Gag peptides was 295 SFC/10<sup>6</sup> PBMC (range 80–488) after the first rFPV booster was administered (Fig. 2B). Despite the low numbers of IFN $\gamma$ -secreting T cells detected after IM DNA vaccination (mean response 13 SFC/10<sup>6</sup> PBMC, range 0–38, to Gag peptides at week 9), a rapid rise within 1 week of the IM rFPV booster demonstrated effective priming of the cellular response. Similarly, by ICS, high peak T cell responses to pooled SIV Gag 15mer peptides in both CD4<sup>+</sup> (mean 0.61%, week 14 Fig. 2C) and CD8<sup>+</sup> (mean 2.33%, week 16 Fig. 2D) T cell populations were generated early following the IM rFPV boost in IM DNA primed animals. A second rFPV boost failed to enhance SIV-specific immunity, possibly related to the generation of immune responses against the FPV vector [29].

Neither mucosal (IN) delivery of the DNA or rFPV vaccines nor mucosal (IN or IR) boosting with the rFPV vaccines in animals primed systemically with DNA vaccine induced systemic T cell immunity as measured in peripheral blood. T cell responses were not detected either by IFN $\gamma$  ELISpot or ICS in these groups above levels seen in control-vaccinated animals (Fig. 2). In addition, there were no significant memory SIV Gag-specific CD4 or CD8 T cell responses as assessed by CFSE proliferation studies and flow cytometry (Fig. 3).

### 3.3. SHIV<sub>SF162P3</sub> challenge

To analyze the protective efficacy of these vaccine regimens, we utilized a CCR5-tropic vaginally-delivered

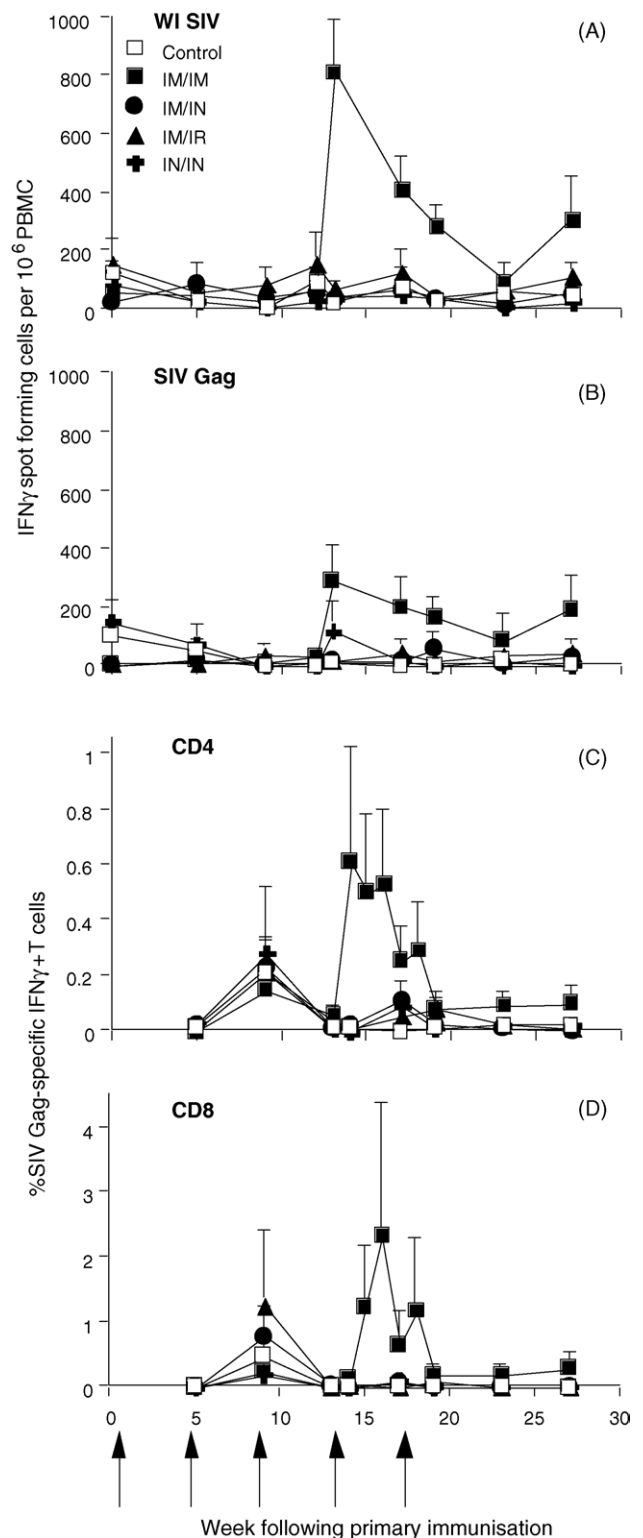


Fig. 2. SIV-specific cell immunity by IFN $\gamma$  ELISpot and ICS. (A) Mean IFN $\gamma$ -producing spot forming cells (+S.E.) to whole inactivated SIV (WI SIV) and to (B) a SIV Gag 15mer peptide pool over the course of the DNA and rFPV vaccinations are shown for each vaccine regimen as outlined in Table 2. Gag specific (C) CD4 and (D) CD8 T cell responses by ICS (mean + S.E.) prior to and following the rFPV boost is shown for each vaccine regimen.

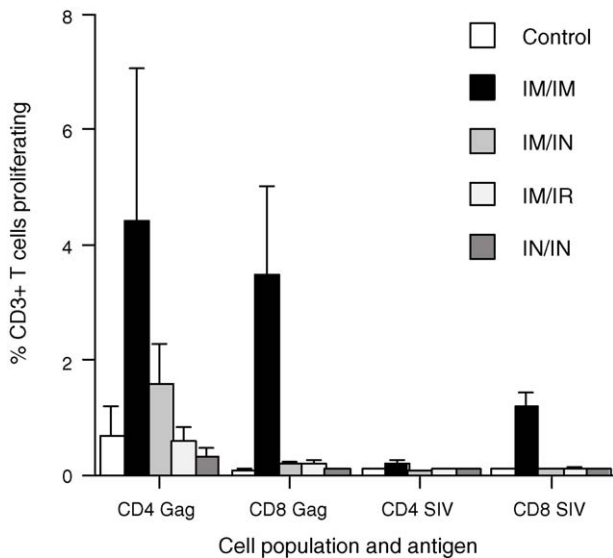


Fig. 3. Lymphoproliferative responses post-immunisation. CFSE labelled PBMC obtained following rFPV boosting were incubated with either SIV Gag peptides or whole inactivated SIV, and the corresponding controls (DMSO and microvesicles, respectively). Proliferating PBMC were phenotyped for CD4 and CD8 lymphocytes. Mean (+S.E.) proliferation responses are shown for each group post-immunisation with the rFPV vaccines.

SHIV challenge system to mimic the most common mode of mucosal heterosexual transmission of HIV-1. The SHIV<sub>SF162P3</sub> challenge stock was inoculated atraumatically into the vagina ( $6 \times 10^3$  TCID<sub>50</sub> over 2 days) in all 24 macaques at 10 weeks after the last rFPV booster vaccination. CD4 T cell numbers and plasma SHIV RNA were followed for 11 weeks following challenge, at which time all vaccinated animals were euthanased and samples obtained for assessment of mucosal T cell responses (Fig. 4). Since SHIV<sub>SF162P3</sub> has shown considerable variability in the long-term outcome of challenge [33–35,39], the primary outcome of challenge in these experiments was determined by a time-weighted, area under the curve analysis of SHIV plasma measured twice weekly during the acute infection period until 4 weeks following challenge.

The eight control macaques that did not receive SHIV-expressing DNA and FPV vaccines had high peak levels of SHIV RNA (mean  $7.52 \log_{10}$  copies/mL, range  $6.76$ – $8.40 \log_{10}$  copies/mL) following inoculation, and at set point (weeks 6–11; mean  $5.19 \log_{10}$  copies/mL, range  $<3.2$ – $6.88 \log_{10}$  copies/mL; Fig. 4A). These animals lost a

proportion of their peripheral CD4 T cells during the acute infection period, the numbers declining to a mean of 15.2% of lymphocytes (range 8.5–24.7%) by week 3.5 (Fig. 4; corresponding to a 42% mean reduction from baseline). Three of the eight control macaques appeared to control SHIV viremia to low levels ( $<3.2 \log_{10}$  copies/mL) by 11 weeks after infection.

All macaques used in the study became infected with SHIV<sub>SF162P3</sub> following the vaginal challenge (Fig. 4B–F, left panels). Two of the macaques in the IM/IM group died of causes unrelated to vaccinations or the SHIV challenge (bowel obstructions), one prior to challenge, and the other 2 weeks after challenge. The animal that died following challenge had a low ( $4.55 \log_{10}$  RNA copies/mL) level of plasma SHIV viremia and no reduction in circulating CD4 T cells at the time of death.

Despite the low level of circulating T cell responses in mucosally-vaccinated macaques prior to vaginal SHIV challenge, there was a statistically significant five-fold mean reduction in acute SHIV<sub>SF162P3</sub> RNA in plasma from the IM/IR immunized group in comparison to controls in the primary endpoint analysis of the time-weighted area-under-the-curve analysis ( $p=0.027$ , rank sum test, Fig. 4, Table 3). Although some reductions in acute SHIV RNA levels in the other mucosally immunized groups were recorded, these were not statistically significant. In addition, a 10-fold reduction in the acute SHIV RNA levels was found in the IM/IM group, although this failed to achieve statistical significance ( $p=0.068$ ), most likely due to the unrelated deaths in two animals in that group.

As a secondary statistical analysis, we also looked at peak acute SHIV VL. Peak plasma SHIV VL was significantly reduced by  $0.9 \log_{10}$  copies/mL in both the IM/IR and IM/IN groups in comparison to controls (both  $p=0.042$ , Table 3). Although the study was not powered to look at long term virological or CD4 T cell outcomes, all four animals in the IM/IR group and both remaining animals in the IM/IM group exhibited low ( $<3.5 \log_{10}$  copies/mL) SHIV VL levels by week 11, in comparison to only three of eight controls, two of four in the IM/IN group and one of four in the IN/IN group.

### 3.4. Immune responses following SHIV challenge

To assess priming of protective immune responses in our vaccine groups, SHIV-specific immune responses were analyzed following SHIV<sub>SF162P3</sub> challenge, firstly in the periph-

Table 3  
Statistical analysis of viral load after challenge

Group	N	Mean TWAUC acute SHIV RNA <sup>a</sup>	p-value vs. controls	Mean peak acute SHIV RNA	p-value vs. controls
Controls	8	5.82	–	7.69	–
IM/IM	2 <sup>b</sup>	4.82	0.068	7.05	0.12
IM/IN	4	5.36	0.28	6.81	0.042
IM/IR	4	5.07	0.027	6.83	0.042
IN/IN	4	5.37	0.24	7.43	0.45

<sup>a</sup> Time-weighted area-under-the-curve (TWAUC) analysis of SHIV plasma RNA ( $\log_{10}$  copies/mL plasma) during the first 4 weeks following challenge.

<sup>b</sup> This group was smaller owing to the two unrelated deaths in animals in this group.

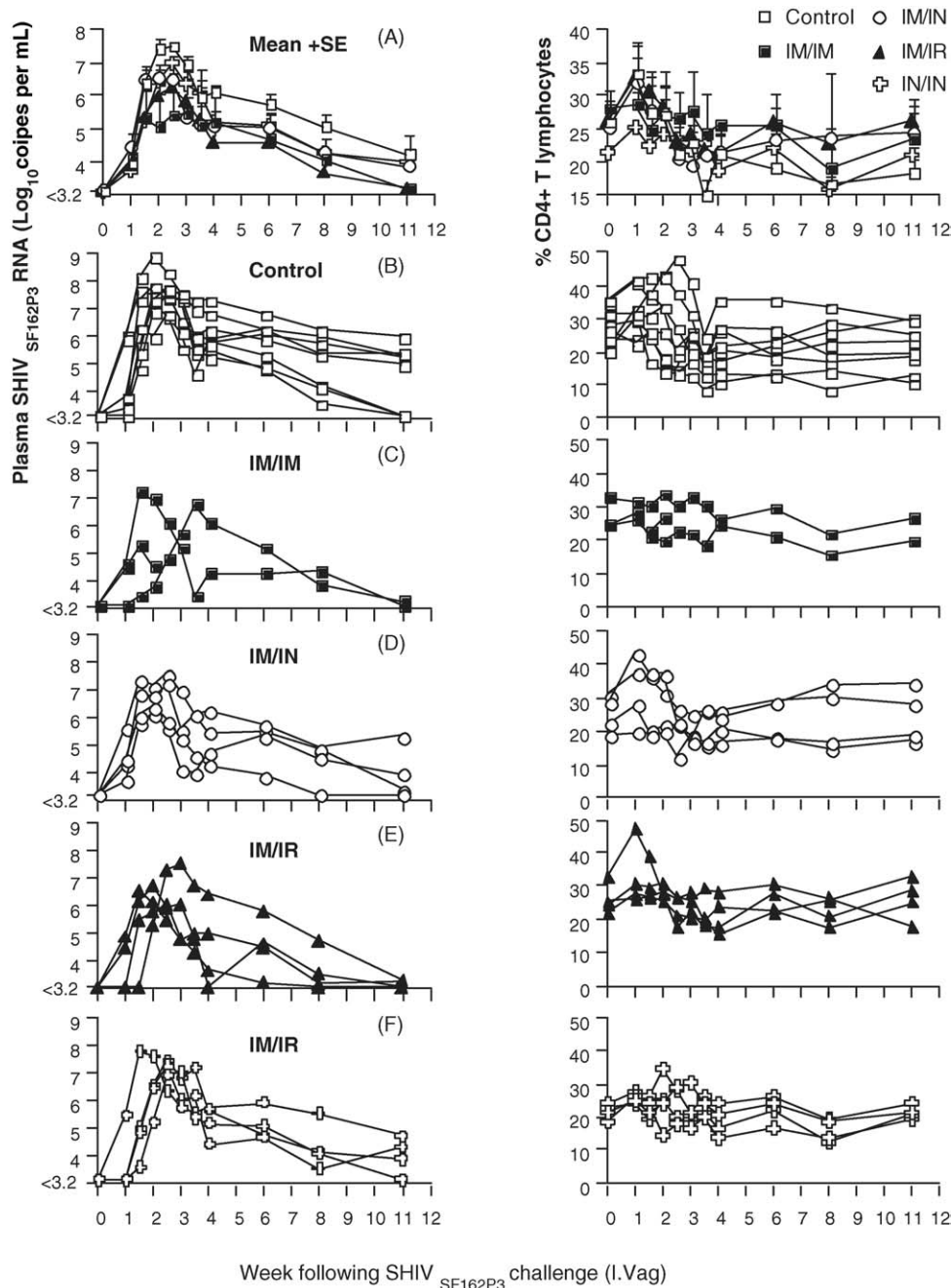


Fig. 4. Outcome of SHIV<sub>SF162P3</sub> challenge. Each macaque was challenged intravaginally with  $6 \times 10^3$  TCID<sub>50</sub> SHIV<sub>SF162P3</sub> 10 weeks post final immunizations. Blood samples were analyzed for plasma SHIV RNA by real-time PCR and for CD4 T cell loss until autopsy at week 11 following challenge. (A) Mean (+S.E. of the mean) plasma SHIV RNA and CD4 T cells for each vaccine group. (B–F) Plasma SHIV RNA and CD4 T cell responses are shown for individual macaques grouped for each vaccine regimen.

eral circulation by IFN $\gamma$  ICS, ELISpot and NAb studies (Fig. 5). SIV Gag-specific T cells expressing IFN $\gamma$  were boosted after challenge in all groups when analyzed by either ELISpot or ICS (Fig. 5A–D). Mean Gag-specific T cell numbers by ELISpot peaked at 2.5 weeks following challenge at 736 SFC/10<sup>6</sup> PBMC (range 30–1443) in the macaques immunized via the IM/IM regimen (Fig. 5B). The other vaccine groups had mean post-challenge peak T cell ELISpot responses numbering 578 (range 15–1625) in the IM/IR

group, 589 (range 70–1735) in the IM/IN group and 114 (range 0–455) in the IN/IN group, the latter being closer to the control group that numbered 223 (range 13–508). A very similar pattern among the vaccine groups was observed when T cell responses were studied using a different viral antigen, whole inactivated SIV (Fig. 5A).

The post-challenge circulating T cell responses were phenotyped by ICS. A substantial increase in IFN $\gamma$ -expressing Gag-specific CD4 and CD8 T cell numbers was also observed



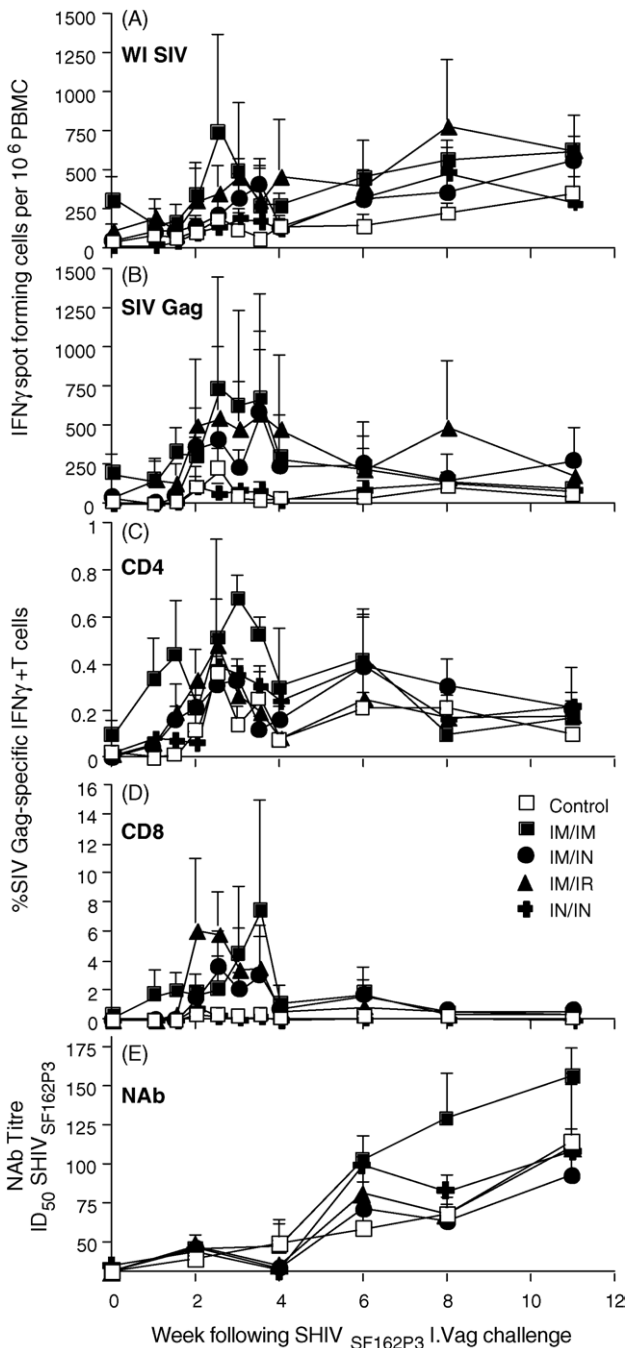


Fig. 5. Immune responses following SHIV challenge. (A) Mean IFN $\gamma$ -producing spot forming cells (+S.E.) to whole inactivated SIV (WI SIV) and to a SIV Gag 15mer peptide pool following SHIV<sub>SF162P3</sub> challenge are shown for each vaccine regimen as outlined in Table 2. SIV Gag specific (C) CD4 and (D) CD8 T cell responses by ICS (mean + S.E.) following challenge. (E) The plasma NAb titer to SHIV<sub>SF162P3</sub> on TZM-bl cells (mean + S.E.) following challenge.

in vaccinated macaques after SHIV challenge (Fig. 5C and D). Peak mean Gag-specific CD4 and CD8 T cell responses were of similar magnitude in the IM/IM group (mean 0.68% and 7.52%, respectively) and the IM/IR group (0.48% and 6.14%, respectively), with CD8 responses peaking earlier in

the animals that were boosted via the mucosal route. The Gag-specific CD8 T cell ICS responses seen in the different vaccine groups were consistent with those measured by ELISpot, with mean responses being highest in the IM/IN, IM/IR and IM/IM groups.

Since partial control of SHIV<sub>89.6P</sub> viremia in previous DNA and poxvirus vaccine studies was associated with a rapid rise in NABs [19], we also assessed NAB responses to SHIV<sub>SF162P3</sub> following challenge. NAB responses were not detected at the time of challenge (Fig. 5E), consistent with the divergent and mutated Env genes encoded in the vaccines. A slow rise in the level of NAB responses to the challenge SHIV<sub>SF162P3</sub> strain emerged at 4–6 weeks following challenge in all groups and NAB levels continued to rise in all groups until autopsy at week 11.

### 3.5. Mucosal T cell immunity following challenge

We endeavoured to measure post-vaccination mucosal T cell responses by ELISpot and ICS using cells isolated from rectal and endocervical biopsies taken prior to challenge. Mononuclear cell isolates from rectal biopsy samples again showed high levels of spontaneous IFN $\gamma$  production, while endocervical biopsies taken prior to autopsy yielded too few T cells to provide useful information in these assays. It is becoming clear that analyses of non-human primate T cell responses from mucosal tissues where activation status and very limited numbers are problematical may greatly be facilitated by the availability of inbred animals that allow tetramer staining of SIV epitope-specific CD8<sup>+</sup> T cells in Mamu-A\*01 positive rhesus macaques [25,27] or even in pigtail macaques [40], although neither option was available to us in the present study.

At autopsy, 11 weeks following SHIV<sub>SF162P3</sub> infection, we were able to obtain larger numbers of T cells from rectal mucosa and endocervical tissues. We modified our standard IFN $\gamma$  ELISpot assay to rest the cells in culture for 24 h prior to antigen stimulation and this successfully reduced background IFN $\gamma$  production. Interestingly, animals immunized via the mucosal regimens (IM/IN, IM/IR, IN/IN) had greater numbers of IFN $\gamma$ -secreting SIV Gag-specific T cells present in the endocervix (Fig. 6A); indeed, the mean number found in the three mucosal groups was 968 SFC/10<sup>6</sup> cells in comparison to 327 SFC/10<sup>6</sup> in the controls and systemically immunized animals ( $p=0.03$ , rank sum test). The patterns of rectal mucosal T cell responses were somewhat different, with animals in the mucosal IM/IR and IN/IN groups, and the IM/IM group, generating the highest mean responses (Fig. 6B). The strongest genital and rectal mucosal T cell responses were found in animals that were DNA-primed IM and rFPV-boosted intrarectally, although there was considerable variability between outbred animals within each of the vaccine groups. The mean and range of the ELISpot responses (SFC/10<sup>6</sup> cells) and number of animals studied at each time for the rectal cells was: controls 123, 0–390,  $n=4$ ; IM/IM 353, 200–575,  $n=4$ ; IM/IN 147, 0–520,  $n=4$ ; IM/IR 422, 0–1180,  $n=4$ ; IN/IN

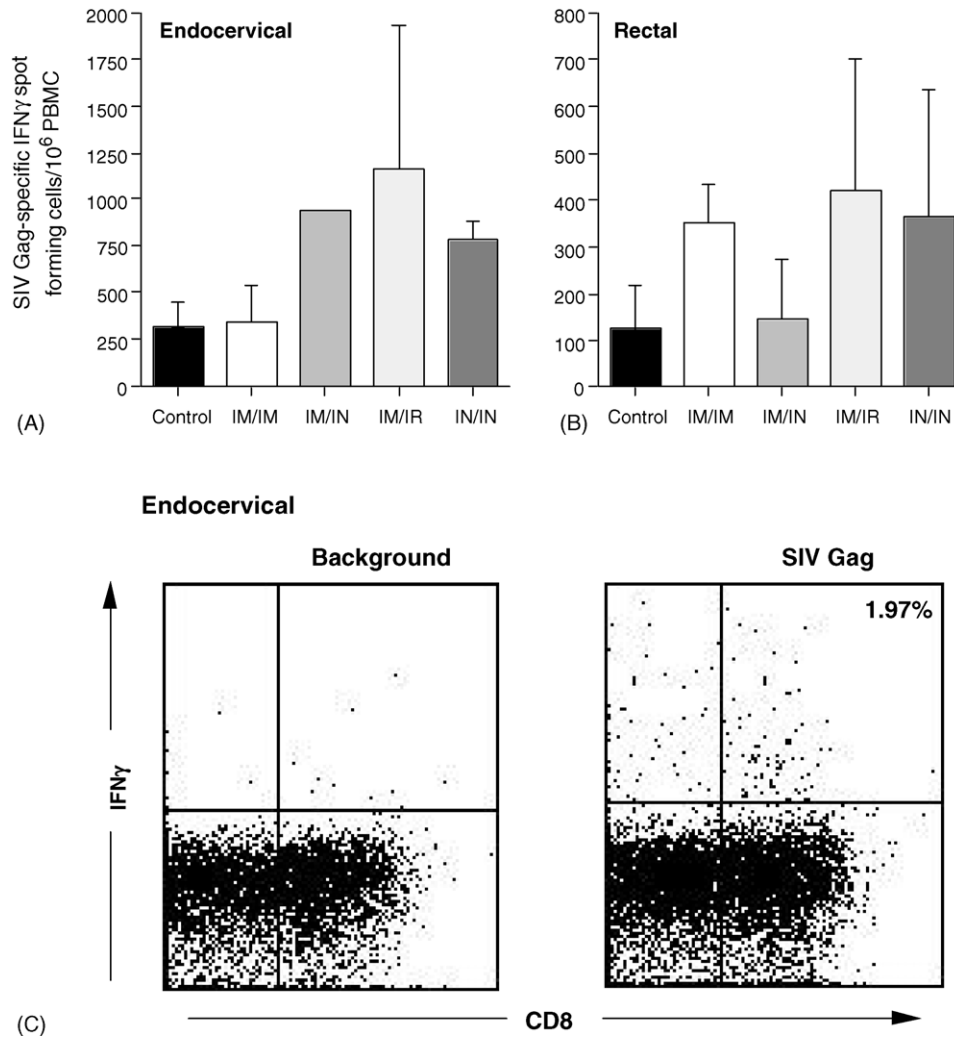


Fig. 6. Mucosal T cell immunity following SHIV challenge. At autopsy, (A) endocervical and (B) rectal mononuclear cells were isolated, rested for 24 h and assayed by IFN $\gamma$  ELISpot for SIV Gag overlapping peptide-specific T cell responses (mean + S.E.) across the vaccine groups. (C) Endocervical Gag-specific T cells in one of the IM/IR vaccinated animals was phenotyped by ICS, with ~2% of cells specifically expressing IFN $\gamma$  in response to SIV Gag peptide stimulation (right panel). Background (DMSO) stimulated endocervical mononuclear cells are shown in the left panel.

366, 0–1160,  $n=4$  and for the endocervical cells: controls 319, 65–487,  $n=3$ ; IM/IN 940,  $n=1$ ; IM/IR 1153, 122–3472,  $n=4$ ; IN/IN 789, 575–1055,  $n=4$ .

The SIV Gag-specific endocervical T cell isolates from one animal in the IM/IR group were phenotyped by ICS and the majority of responding cells were CD3<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 6C). Gag-specific T cells represented 2% of the endocervical CD8 T cells in this animal.

#### 4. Discussion

We tested the immunogenicity and protective efficacy of mucosally-delivered DNA and rFPV prime and boost vaccines against a virulent mucosal (vaginal) CCR5-utilizing SHIV challenge in non-human primates. Mucosal boosting with rFPV via either IR or IN routes in macaques that had been primed systemically (IM) with DNA vaccines induced

recall CD4 and CD8 T cell immunity to SHIV and a modest but significant reduction in acute SHIV viral loads. Interestingly, these animals had poor circulating T cell responses prior to challenge with SHIV compared to those primed and boosted via the intramuscular route. In contrast to the promise of the IM/IR and IM/IN vaccine regimens, a DNA/rFPV vaccine regimen based wholly on intranasal delivery was not protective in macaques. It may be that further adjuvants are required to increase the efficacy of nasal delivery of these constructs in macaques [27]. Our data indicate that effective immune priming for partial protection against pathogenic mucosal challenge was achieved with these systemic DNA prime and mucosal rFPV boost vaccination strategies.

To assess the protective efficacy of our vaccine strategies, we utilized a detailed kinetic analysis of acute SHIV viral levels, taking samples every 3–4 days during acute infection to overcome the variable long term outcome of SHIV<sub>SF162P3</sub> infection in macaques [33–35,39]. Significantly, the Env of

SHIV<sub>SF162P3</sub> is derived from a CCR5-tropic primary HIV-1 isolate and the vaginal infection model we studied likely represents a good approximation of male to female HIV-1 transmission in humans [28]. Many vaccine studies to date have used intra-rectal challenge with CXCR4-utilizing SHIV strains such as SHIV<sub>89,6P</sub> that target naïve rather than memory CD4 T cells [28]. Similar to natural infection in humans, and in contrast to SHIV<sub>89,6P</sub> challenge studies, high levels of homologous NAb responses to SHIV<sub>SF162P3</sub> were not generated until after the acute infection period following challenge. The slow generation of NAb to SHIV<sub>SF162P3</sub> is consistent with the heterologous nature of the neutralization determinants in the vaccines, and the primary, CCR5-tropic HIV-1<sub>SF162P3</sub> Envelope in the SHIV challenge stock. This enabled the primary analysis of T cell responses in protective immunity, the primary goal of our DNA/rFPV regimens, given that envelope immunogens that induce broad and effective NAb remain elusive.

The efficacy of a protective vaccine against HIV-1 will depend, at least in part, on its ability to induce long-term mucosal immune responses and/or those that are rapidly activated upon local exposure to the virus. After infection, the time period during which local immune responses could potentially contain or even eradicate the virus before widespread systemic dissemination are likely to be of the order of a few days [41]. The protection that we observed in our systemic prime/IR mucosal boost vaccine group was intriguing. Little or no SHIV-specific circulating CD4 or CD8 T cell responses were induced by either the IM/IR or IM/IN vaccination regimen as measured by IFN $\gamma$  ELISpot, intracellular cytokine staining, or proliferation studies. However, following challenge, sharp increases in the magnitude of CD8<sup>+</sup> T cell responses were observed in the IM/IR and, to somewhat lesser extent, in the IM/IN vaccination group (similar to those observed in the systemically vaccinated IM/IM group). Although the “priming” effect of the IM DNA vaccine alone may have been sufficient to recall effective immune responses following challenge, the mucosally delivered rFPV may have contributed to enhanced protective immunity [22]. In our recent murine studies of these vaccine strategies, mucosal IR or IN rFPV boosting was required for expression of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses after systemic (IM) DNA priming, notably in the lymph nodes draining the genital and rectal mucosa (Ranasinghe et al., unpublished data). In this context, the analyses of macaque mucosal CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses performed at autopsy, i.e. after vaginal challenge, were interesting, demonstrating enhanced endocervical T cell responses in the mucosally-immunized animals in comparison to the systemically (IM/IM) immunized animals and controls. These data raise the possibility that more effective priming of genital tract immunity occurred following mucosal administration of the rFPV booster vaccine. Interpretation of these mucosal T cell responses should be tempered with the small numbers of animals in the outbred pigtail macaque groups and the knowledge that many of the responses are likely to reflect the disseminated SHIV

infection, rather than the priming of responses induced by the vaccines alone. The functional role of mucosal T cells later following virus exposure is likely to be diminished in comparison to responses present or generated within the first few days of challenge. Further studies in macaques are warranted involving detailed kinetic studies of mucosal T cell responses following vaccination by these routes and pathogenic mucosal challenge. Multiple “real-time” analyses of mucosal T cell immunity would be greatly improved by the use of tetramer staining of SIV epitope-specific CD8<sup>+</sup> T cells in inbred Mamu-A\*01 rhesus macaques [25,27]. We are now generating MHC class I tetramers to common SIV epitopes in pigtail macaques to facilitate such studies [40].

Effective uptake of vaccine antigens at mucosal surfaces is problematical and has long represented a challenge for mucosal vaccine development. The use of alternative vector systems, including those with demonstrated tropism for mucosal surfaces, may help increase the uptake and presentation of encoded vaccine antigens at mucosal surfaces [15,16]. We are currently evaluating mucosal delivery of recombinant adenovirus vectors and recombinant vaccinia virus/rFPV prime-boost combinations as mucosal vaccine regimens in mice (Ranasinghe, unpublished data; Ramsay, unpublished data). Vaccine constructs based on vaccinia and adenovirus may well prime for superior mucosal immunity or boost mucosal responses through their capacity for high-level replication in mucosal tissues.

In conclusion, boosting of IM DNA primed macaques with rFPV delivered by the IR mucosal route, primed recall T cell immunity to SHIV that resulted in a significant reduction in acute SHIV viral levels. An important goal is to develop strategies that will induce durable immune responses both at mucosal sites of transmission and in the systemic circulation. Additional refinements to our vaccine regimens and improvements in the technology for mucosal T cell analysis, should provide a rationale for future human trials with the goal of reducing HIV-1 transmission and disease.

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