Induction of mucosal and systemic antibody and T-cell responses following prime–boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations

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As sexual transmission of human immunodeficiency virus-1 (HIV-1) occurs via the mucosa, an ideal HIV-1 vaccine should induce both mucosal and systemic immunity. We therefore sought to evaluate the induction of mucosal responses using a DNA *env* prime–gp120 protein boost approach in which sequential nasal and parenteral protein administration was performed with two novel carbohydrate-based adjuvants. These adjuvants, Advax-M and Advax-P, were specifically designed for mucosal and systemic immune enhancement, respectively. Murine intranasal immunization with gp120/Advax-M adjuvant elicited gp120-specific IgA in serum and mucosal secretions that was markedly enhanced by DNA priming. Boosting of DNA-primed mice with gp120/Advax-M and gp120/Advax-P by sequential intranasal and intramuscular immunization, or vice versa, elicited persistent mucosal gp120-specific IgA, systemic IgG and memory T- and B-cell responses. Induction of homologous, but not heterologous, neutralizing activity was noted in the sera of all immunized groups. While confirmation of efficacy is required in challenge studies using non-human primates, these results suggest that the combination of DNA priming with sequential nasal and parenteral protein boosting, with appropriate mucosal and systemic adjuvants, could generate strong mucosal and systemic immunity and may block HIV-1 mucosal transmission and infection.

INTRODUCTION

Mucosal transmission of human immunodeficiency virus (HIV)-1 often leads to rapid depletion of activated $CD4^+CCR5^+$ T-cells in mucosal tissues and establishes a major reservoir for virus persistence in gut-associated lymphoid tissues (Brenchley *et al.*, 2004; Chase *et al.*, 2007; Pandrea *et al.*, 2007). This indicates that an HIV vaccine should induce strong and long-lasting mucosal immunity at both the B- and T-cell level. Induction of HIV-specific IgA and CTL at critical mucosal sites should provide a first line of defence to block mucosal penetration, with systemic HIV-specific IgG and CTLs defending against parenteral HIV-1 transmission (Belyakov *et al.*, 2006; Shattock *et al.*, 2008; Srivastava *et al.*, 2008; Vajdy, 2006).

Limited HIV-1 vaccine studies have shown that mucosal immunization can elicit secretory IgA, CTL and memory B- and T-cell responses in mucosal compartments that are dependent on the vaccine regimen, route of immunization

and adjuvants used (Alving & Rao, 2008; Lai *et al.*, 2007; Manrique *et al.*, 2009; Vajdy & Singh, 2006). Mucosal adjuvants that have been studied extensively include the secreted enterotoxins of *Vibrio cholerae* and *Escherichia coli*, and mutated forms thereof [e.g. cholera toxin, Escherichia coli heat-labile toxin (LT), LTK63 (non-toxic LT mutant) and LTR72 (non-toxic LT mutant)] (Connell, 2007; Glenn *et al.*, 2007; Stevceva & Ferrari, 2005). However, the use of these toxins as mucosal adjuvants has been impeded by safety issues, most notably cases of facial palsy in human trials of an LT-adjuvanted nasal influenza vaccine (Couch, 2004; van Ginkel *et al.*, 2000, 2005). Many parenteral adjuvants such as CpG oligodeoxynucleotides, polymerized liposomes, microparticles and interleukins [such as IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF)], are currently being evaluated as mucosal adjuvants. However, none of these appear to be as successful as CT or LT in induction of mucosal immunity (Ahmed *et al.*, 2005; Bradney *et al.*, 2002; Manrique *et al.*, 2008; Matyas *et al.*, 2009; Staats *et al.*, 2001). Until recently, relatively little attention has been given to the potential use

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of carbohydrate compounds as mucosal adjuvants. Two highly promising carbohydrate-based adjuvant systems currently in advanced-clinical and preclinical testing are Advax-P and Advax-M, respectively. These adjuvants are derived from natural sugar-containing compounds extracted from plants (Advax-P) and marine sponges (Advax-M) that have potent immune-enhancing activities (Fujii *et al.*, 2006; Kobayashi *et al.*, 1995; Petrovsky, 2006), and have favourable safety profiles in animal models and humans (Cooper, 1995; Cooper *et al.*, 1991; Veldt *et al.*, 2007). Advax-P is a microparticulate adjuvant formulation based on δ-inulin and is specifically designed for parenteral administration. Advax-P has previously proved successful for enhancing neutralizing immune responses against Japanese encephalitis virus (Lobigs *et al.*, 2010) and seasonal and pandemic H1N1/2009 influenza virus (N. Petrovsky, personal communication), while exhibiting good tolerability. In contrast, Advax-M is a glycolipid adjuvant formulation based on α-galactosyl ceramide and is specifically designed for mucosal administration. Galactosyl ceramide and its analogues are potent natural killer T-cell agonists, enhance mucosal IgA production via a mechanism dependent on interleukin (IL)-4 and have been shown to enhance protection against heterologous influenza-virus challenge when nasally administered with an inactivated influenza antigen (Kamijuku *et al.*, 2008).

The hypothesis of the current study is that induction of optimal mucosal and systemic immunity to HIV-1 may require a multimodal vaccine approach. Initial DNA immunization would maximize helper T-cell and B_{mem} priming. Subsequent intranasal (IN) and parenteral immunization with protein, using two novel adjuvants, would boost systemic and mucosal responses. Mucosal and systemic immune responses were evaluated in mice following priming with DNA encoding HIV-1 envelope (*env*) and boosting with gp120 protein combined with either Advax-M or Advax-P adjuvants delivered via mucosal, parenteral or mucosal/parenteral combination routes. Results presented here demonstrate that this DNA prime–protein boost vaccine-regimen strategy, incorporating Advax-M and/or Advax-P adjuvants, elicits robust and durable immune effector and memory responses in both mucosal and systemic compartments and may therefore contribute to enhanced protection against HIV-1.

RESULTS

Generation of systemic and mucosal immune responses following DNA priming and protein boosting by mucosal or parenteral routes

To provide a baseline adjuvant comparison, BALB/c mice were immunized with recombinant-HIV-1$_{Ba-L}$ gp120 formulated in two novel adjuvants, Advax-M and Advax-P1, delivered by IN and intramuscular (IM) routes, respectively (Fig. 1a). Anti-gp120 IgG was measured in serum and anti-gp120 IgA was measured in serum, saliva and vaginal wash samples. Serum anti-gp120 IgA titres were detected in mice immunized with Advax-M-formulated gp120 but not with Advax-P1-adjuvanted gp120 ($P<0.01$; Fig. 2a, upper panel). A similar trend was noted for pooled saliva from test groups, in which IgA was only detected in mice immunized with gp120/Advax-M (Fig. 2a, lower panel). For vaginal wash samples, anti-gp120 IgA was not detectable in any groups that had been immunized with adjuvanted protein alone (Fig. 2a, middle panel). Anti-gp120 IgG responses were noted in both test groups and were found to be greater in the Advax-P1 group compared with the Advax-M group (data not shown).

Earlier studies in mice and macaques demonstrated that, in DNA-primed animals boosted parenterally with QS-21-adjuvanted protein, systemic IgG and T-cell responses were induced (Cristillo *et al.*, 2006; Pal *et al.*, 2005, 2006). A
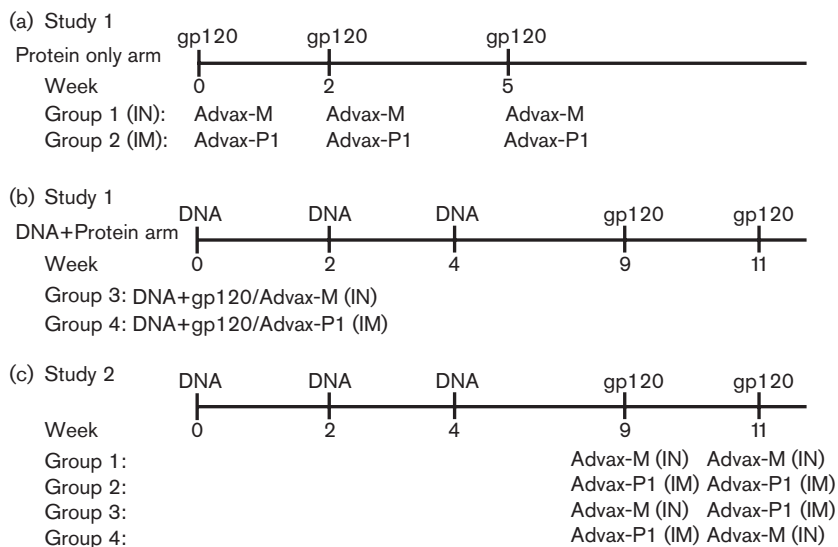

Fig. 1. Murine immunization schedule. (a) Mice (*n*=5) were immunized with recombinant gp120 (25 μg) via the IM route for Advax-P1 (1 mg) or via the IN route for Advax-M (2 μg) at weeks 0, 2 and 5. (b) Alternatively, mice (*n*=5) were immunized with DNA (100 μg) at weeks 0, 2 and 4, and with adjuvanted gp120 protein at weeks 9 and 11. (c) Mice (*n*=10) were immunized intramuscularly with DNA (100 μg) at weeks 0, 2 and 4 and boosted with adjuvanted gp120 (25 μg) for either Advax-M or Advax-P1 via the IN or IM routes, respectively, at weeks 9 and 11. Combination delivery strategies were tested in which gp120 was administered by the IM route for Advax-P1 at week 9 and by the IN route for Advax-M at week 11 or vice versa.
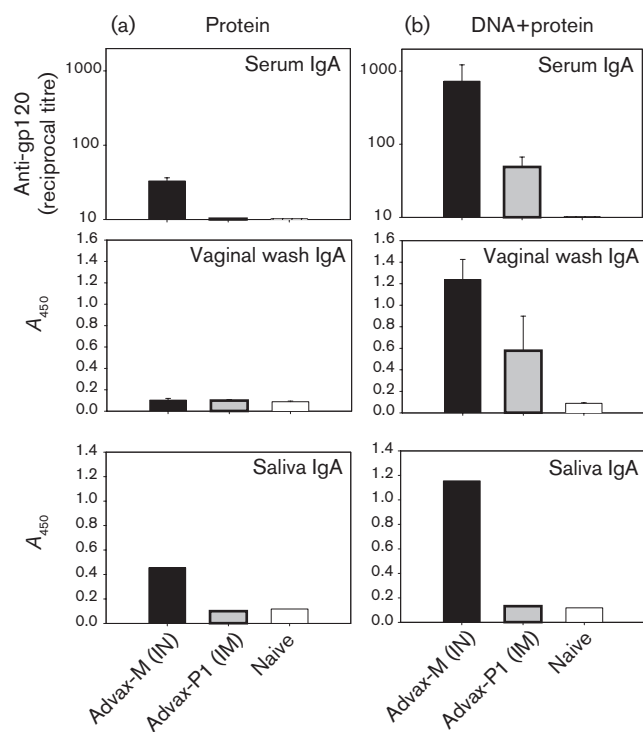
Fig. 2. Generation of anti-gp120 IgA antibodies in serum and mucosal compartments of mice immunized with DNA and adjuvanted protein. At 2 weeks post-protein immunization, anti-gp120 IgA responses, assayed by ELISA, were determined for serum (upper panels), vaginal wash samples (middle panels) and saliva (lower panels) following protein (a), and DNA prime–protein boost (b), immunization. Mean serum titres ± SEM values are shown. For vaginal-wash samples (1 : 2 dilution) and saliva (1 : 5 dilution), mean A_{450} measurements ± SEM are shown for the diluted samples.

similar DNA prime–protein boost vaccine regimen was used here to compare mucosal and systemic immune responses elicited by Advax-M and Advax-P1 given via IN and IM routes, respectively. Mice primed with three administrations of DNA were boosted twice with adjuvanted gp120 by IN or IM routes (Fig. 1b). Serum anti-gp120 IgA levels increased in all test groups relative to adjuvanted

protein-only immunization ($P<0.01$) and were greatest in the Advax-M group (Fig. 2b, upper panel). Whereas vaginal wash samples from mice immunized with adjuvanted protein showed no detectable anti-gp120 IgA, antibody levels were markedly augmented in Advax-M and Advax-P1 groups following prime–boost immunization ($P<0.05$) (Fig. 2b, middle panel). When evaluated further in pooled saliva, anti-gp120 IgA responses were only detected in the Advax-M-boosted groups (Fig. 2b, lower panel).

Increased serum-anti-gp120 IgG titres were noted in all adjuvant groups following DNA prime–protein boost compared with protein-only immunization (data not shown). Serum antibody responses, characterized by IgG subclasses (Table 1), revealed that immunization with DNA or protein alone resulted in a predominantly IgG1 response. DNA prime–protein boost immunization incorporating Advax-M or Advax-P1 resulted in a broader response including IgG1, IgG2a and IgG2b, with minimal IgG3 responses detected in the Advax-P1 group. The relative proportions of specific IgG isotypes elicited following prime–boost immunization varied depending upon the adjuvant used. Advax-M yielded profiles in which IgG1>IgG2b>IgG2a and Advax-P1 generated responses in which IgG1>IgG2a>IgG2b.

Mucosal and systemic responses observed in the Advax test groups were compared with previous results obtained with QS-21. No IgA responses were detected in mucosal washes of mice immunized with QS-21-adjuvanted gp120 or following DNA prime–protein boost immunization. In sera, minimal IgA responses were only detected after DNA prime–protein boost immunization (data not shown).

Th1 cytokine responses were noted in mice immunized with gp120/Advax-M, with lower cytokine levels ($P<0.01$ vs Advax-M) observed in mice immunized with gp120/Advax-P1 (Fig. 3a). For Th2 cytokines, IL-5 was elicited in mice immunized with protein/Advax-M and to a lesser extent with protein/Advax-P1 ($P<0.01$) (Fig. 3a). IL-4 levels were either minimal or not detected in both groups. Following prime–boost immunization (Fig. 3b), Th1 cytokine levels increased in both groups relative to adjuvanted protein-only immunization. For the Advax-M group, tumour necrosis factor α (TNF-α), gamma interferon

Table 1. Anti-gp120-specific IgG titres

Median values ± SEM of anti-gp120 IgG-isotype reciprocal titres.

| Vaccine/route	IgG1	IgG2a	IgG2b	IgG3
DNA (IM) only	23 040 ± 6271	980 ± 387	715 ± 415	25 ± 8
gp120/Advax-M (IN)	10 880 ± 1920	10 ± 10	440 ± 147	<25
gp120/Advax-P1 (IM)	28 160 ± 9406	60 ± 37	920 ± 582	<25
DNA + gp120/Advax-M (IN)	204 800 ± 153 600	21 920 ± 11 962	56 960 ± 37 868	<25
DNA + gp120/Advax-P1 (IM)	81 920 ± 30 720	24 320 ± 11 113	8480 ± 2673	170 ± 158
DNA + gp120/Advax-P1 (IM) + gp120/Advax-M (IN)	104 960 ± 41 358	5960 ± 2837	2720 ± 480	<25
DNA + gp120/Advax-M (IN) + gp120/Advax-P1 (IM)	327 680 ± 122 880	62 120 ± 37 351	14 720 ± 9308	180 ± 155
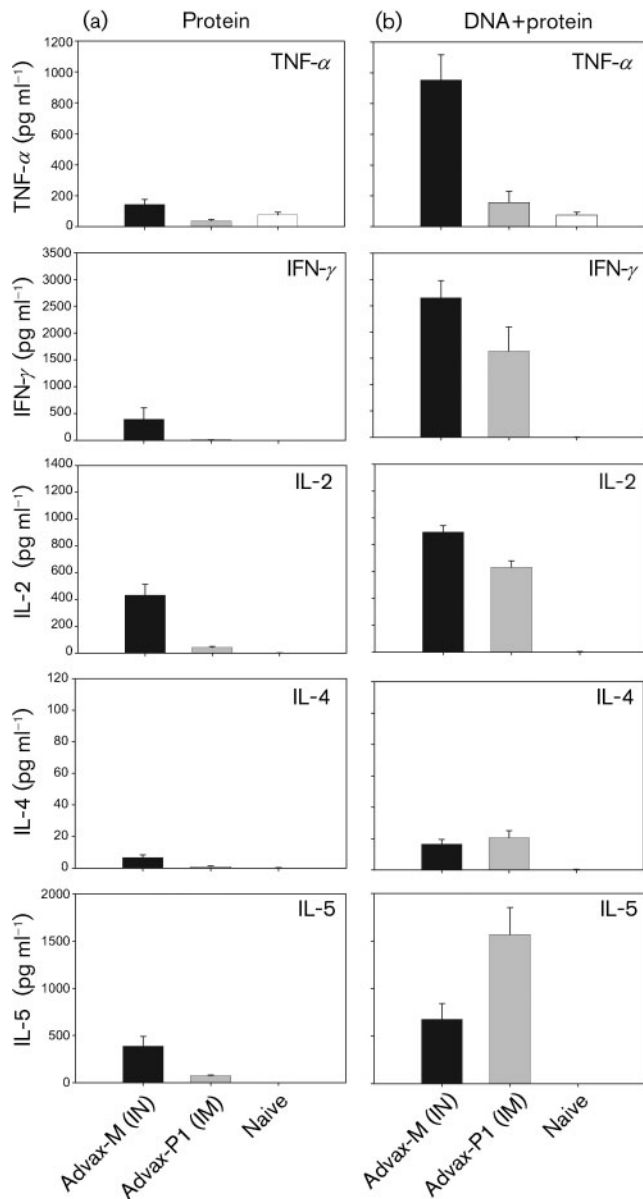
Fig. 3. T-cell responses in splenocytes of mice immunized with DNA and adjuvanted protein. Secreted Th1 (TNF-α, IFN-γ and IL-2) and Th2 (IL-5 and IL-4) cytokines were quantified, by CBA, following a 24 h *ex vivo* stimulation of splenocytes with 1 μg Env peptide pool ml^{-1}, from protein-immunized (a) and DNA prime–protein boosted (b) mice. Mean cytokine responses for each group ± SEM values are shown.

(IFN-γ) and IL-2 were significantly ($P<0.01$) augmented relative to protein-only immunization. For the Advax-P1 group, increased cytokine levels were only statistically significant for IFN-γ and IL-2 ($P<0.01$). Consistent with the Th1 cytokines, a trend of greater Th2 cytokine levels was noted following prime–boost immunization compared with protein-only immunization (Fig. 3b). This increase in Th2 cytokines was statistically significant in the Advax-P1 but not Advax-M group. Mice immunized with DNA followed by QS-21-adjuvanted protein induced comparable Th1 and Th2 cytokines as noted with Advax-P1 (data not shown).

Induction of persistent mucosal- and systemic-anti-gp120 antibodies following DNA prime–protein boost immunization

Given that both systemic and mucosal anti-gp120 IgA responses were seen in mice immunized with Advax-M or Advax-P1, an additional study was conducted to evaluate sequential IN/IM protein boost strategies using these Advax adjuvants in DNA-primed animals. BALB/c mice were immunized with DNA at 0, 2 and 4 weeks and adjuvanted gp120 at 9 and 11 weeks (Fig. 1c). Protein was formulated either in Advax-M or Advax-P1 adjuvant and delivered by IN (IN/IN) or IM (IM/IM) routes, respectively, or in combination (IM/IN, IN/IM). At 2 weeks post-final protein immunization, serum anti-gp120 IgG was found to be comparable in the IM/IM, IM/IN and IN/IM test groups (Fig. 4a, left panel). A trend of lower IgG responses was noted in the IN/IN group but the difference between these levels and those of the other test groups was not found to be statistically significant (Fig. 4a, left panel). To evaluate the decline in titres over time, as previously noted for DNA prime–protein boost immunizations (Pal *et al.*, 2006), anti-gp120 IgG levels were monitored up to week 23 post-boost immunization. Interestingly, anti-gp120 IgG titres increased in all test groups between 2 and 23 weeks post-boost immunization and were comparable in all groups at week 23. Serum antibody responses, characterized by IgG subclass, found broad subclass responses for IM/IN and IN/IM groups, in which IgG1>IgG2a>IgG2b (Table 1). Higher titres for each IgG subclass were seen in the IN/IM group compared with the IM/IN group. IgG3 responses were minimally detected in the IN/IM group but not the IM/IN group, consistent with the asymmetry of the immune response according to the specific order in which IN and IM booster immunizations were administered.

At 2 weeks post-final protein boost immunization (week 13 of the study), anti-gp120 IgA titres were detected in the sera of immunized mice from all test groups and the levels persisted up to 23 weeks post-immunization (week 34 of the study) (Fig. 4a, right panel). A trend for increased serum IgA titres was observed in mice primed with DNA and boosted with adjuvanted protein delivered via the IN/IM route compared with the other test groups; however, this difference was not statistically significant. Anti-gp120 IgA was seen in mucosal samples of immunized mice including pooled saliva (Fig. 4b) and vaginal wash samples (Fig. 4c). Anti-gp120 IgA in the saliva persisted up to 23 weeks post-boost immunization. In vaginal-wash samples, IgA titres were relatively unchanged at 23 weeks post-boost immunization compared to 2 weeks post-boost in all groups.

In order to determine the functional properties of the antibodies elicited by these vaccine regimens, the neutralizing activity of sera collected at 2 weeks following the final
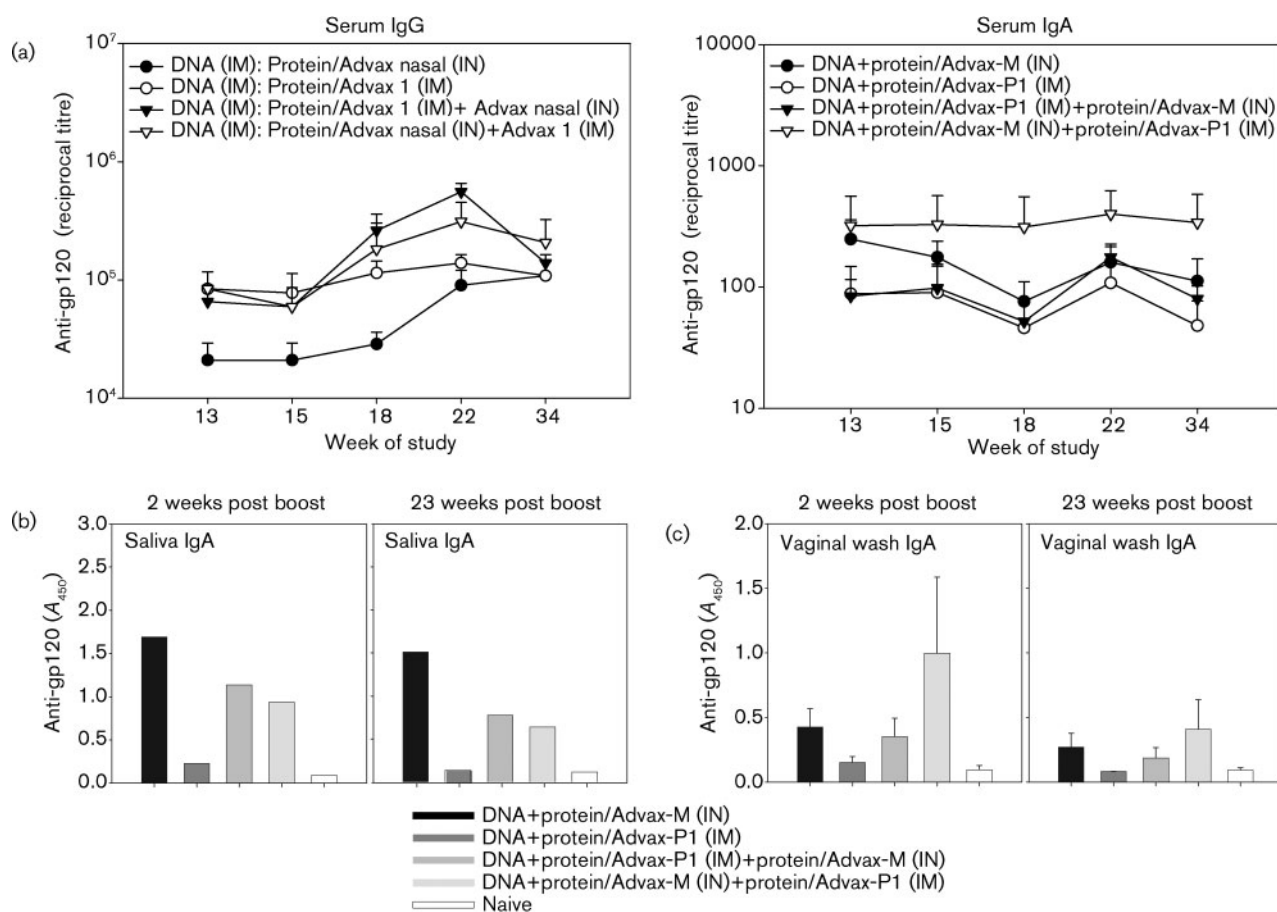
Fig. 4. Durability of anti-gp120 IgG and serum and mucosal IgA responses following immunization of mice via parenteral, mucosal and combination routes. Anti-gp120 IgG (a, left panel) and IgA (a, right panel) responses, assayed by ELISA, were determined for serum samples of DNA prime–protein boosted mice at weeks 13, 15, 18, 22 and 34 of the study. IgA was also measured at weeks 13 and 34 of the study for saliva (b, 1 : 5 dilution) and vaginal-wash samples (c, 1 : 2 dilution). ELISA titres and A_{450} are reported.

immunization was assayed against homologous ($SHIV_{Ba-L}$ and pseudovirus $HIV-1_{BaL.26}$) and heterologous (AC10.0 and QH0692) HIV-1 isolates. Since the volume of serum collected for each mouse was limited, neutralization assays were performed on pooled sera for each immunized group. For these assays, two types of homologous viruses encoding

$HIV-1_{Ba-L}$ envelope were selected. $HIV-1_{BaL.26}$ is a pseudovirus, whereas $SHIV_{Ba-L}$ is a live virus capable of replicating in non-human primates and has been used in previous vaccine-efficacy studies (Pal *et al.*, 2003, 2006). As shown in Table 2, homologous neutralizing activity was detected in all immunized groups against $HIV-1_{BaL.26}$ and

Table 2. Neutralizing activity of sera following immunizations of gp120 with Advax adjuvants

A neutralization assay was conducted with pooled sera from each group of animals. Neutralization titres represent the dilution of serum inhibiting 50 % of infection compared with an untreated control infection. The lowest dilution of serum tested was 1 : 10.

Vaccine/route	BaL.26	$SHIV_{Ba-L}$	AC10.0	QH0692
gp120/Advax-M (IN)	16	70	<10	<10
gp120/Advax-P1 (IM)	27	160	<10	<10
DNA + gp120/Advax-M (IN)	26	100	<10	<10
DNA + gp120/Advax-P1 (IM)	21	92	<10	<10
DNA + gp120/Advax-P1 (IM) + gp120/Advax-M (IN)	10	24	<10	<10
DNA + gp120/Advax-M (IN) + gp120/Advax-P1 (IM)	32	160	<10	<10

SHIV$_{Ba-L}$, with higher titres against SHIV$_{Ba-L}$. Amongst the combination delivery strategies, mice immunized by the IN/IM route elicited higher neutralizing activity compared with the IM/IN route. Heterologous neutralization against AC10 and QH0692 was not detected in any of the immunized groups.

Generation of robust and persistent Th1/Th2 cytokine responses following DNA prime–protein boost immunization

At 2 weeks post-final immunization, gp120-specific IFN-γ-secreting splenocytes were detected in all immunized mice (Fig. 5a, left panel). A trend towards increased IFN-γ was noted in mice primed with DNA and boosted with Advax-M-adjuvanted protein administered via the IN/IN route compared with the other groups. However, this trend was only statistically significant when comparing Advax-M (IN/IN) and Advax-P1 (IM/IM) test groups ($P<0.05$). Th1 (TNF-α and IFN-γ) cytokines, measured by cytometric bead array (CBA) at 2 weeks post-final immunization (Fig. 5b–f, left panel), were comparable in the IN/IN, IM/IN and IN/IM test groups but lower in the IM/IM group ($P<0.05$). In contrast, IL-2 and Th2 (IL-4 and IL-5) cytokine levels were not significantly different between groups. Sustained high levels of gp120-specific IFN-γ-secreting splenocytes were observed at 23 weeks post-final immunization by ELISPOT (Fig. 5a, right panel) and CBA assays (Fig. 5c, right panel). For other Th1 cytokines evaluated, TNFα levels observed at 23 weeks post-immunization were augmented in all test groups relative to the levels at 2 weeks post-immunization (Fig. 5b, right panel). For IL-2, IL-4 and IL-5, levels were markedly enhanced only in mice immunized via IN/IM routes (Fig. 5d–f, right panels).

Generation of memory B- and T-cell responses following DNA prime–protein boost with Advax adjuvants

Persistent levels of anti-gp120 antibodies (Fig. 4) and sustained T-cell responses (Fig. 5) following prime–boost immunization led us to assess memory B- and T-cell responses. Splenocytes from immunized mice (23 weeks post-immunization) were stimulated with either CpG ODN2006 or concanavalin A (ConA), which have previously been shown to activate B$_{mem}$ to become antibody-secreting cells (Guan et al., 2009; Slifka & Ahmed, 1996; Traggiai et al., 2004). Following a 7-day stimulation, anti-gp120 IgG responses were measured in the media (Fig. 6a, b). Under both stimulation conditions, anti-gp120 IgG responses were found to be greater in the IN/IM group relative to the IN/IN or IM/IM groups, but were not significantly different from the IM/IN group. As expected, responses were found to decline with decreasing numbers of splenocytes used. For memory T-cell responses, both CD8 and CD4 effector memory (CD44^{hi}CD62L^{-}; Fig. 6c) and central memory (CD44^{hi}CD62L^{+}; Fig. 6d) T-cells producing Th1 cytokines were observed in all test groups by using
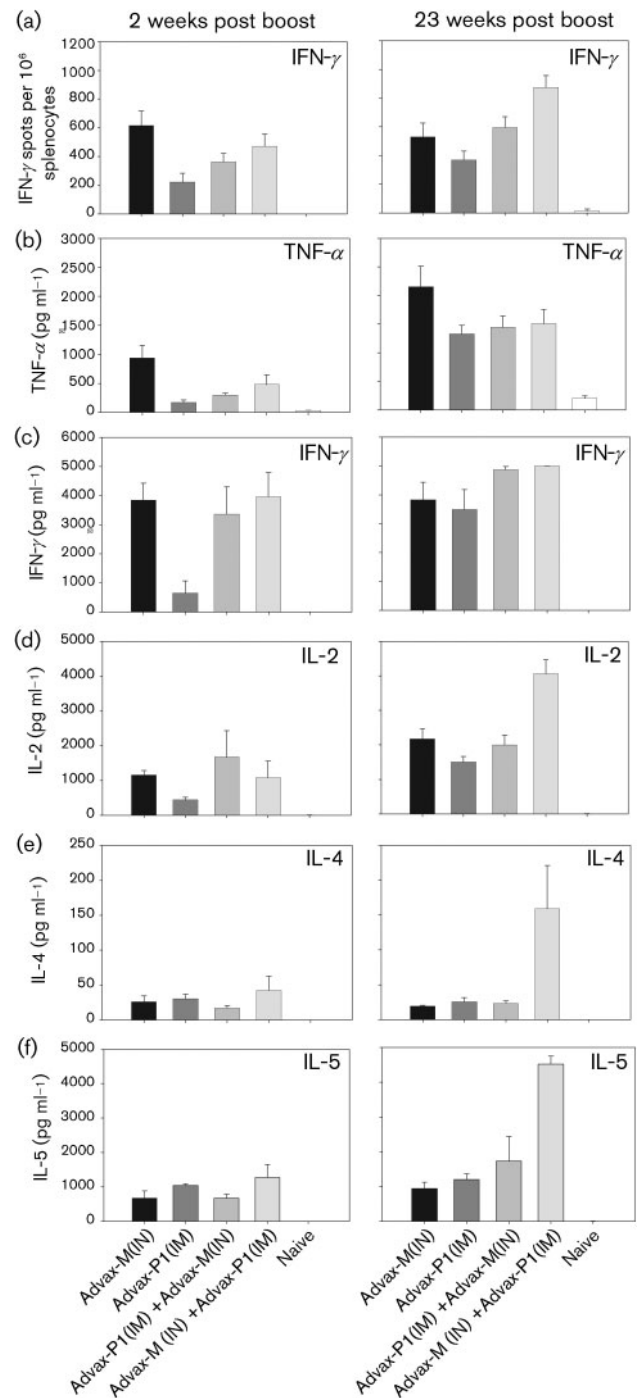

Fig. 5. Durability of gp120-specific Th1 and Th2 cytokine recall responses. Cellular responses were measured, following *ex vivo* Env peptide stimulation, by IFN-γ ELISPOT and CBA assays at 2 (left panels) and 23 weeks (right panels) post-protein boost. IFN-γ production, measured by ELISPOT assay (a), and secreted TNF-α (b), IFN-γ (c), IL-2 (d) IL-4 (e) and IL-5 (f) levels measured by CBA, are shown.

multiparameter FACS analysis. While a trend towards greater cytokine responses was found in DNA-primed mice
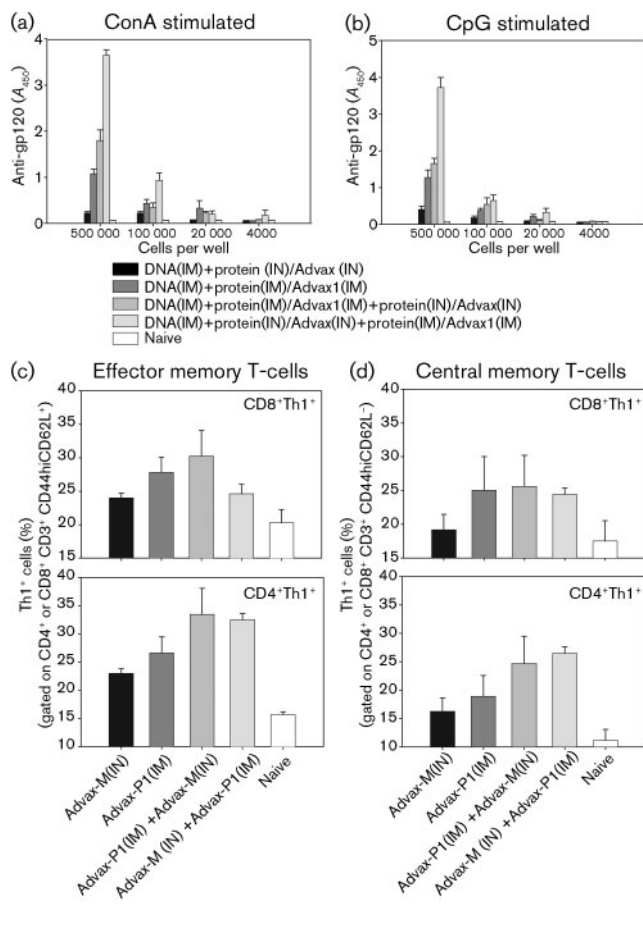
Fig. 6. Generation of B- and T-cell memory following immunization of mice via parenteral, mucosal and combination routes. At 23 weeks post-final immunization, splenocytes (1×10^5, 2×10^4 and 4×10^3) were stimulated for 7 days with a ConA supernatant mixture with mitomycin-treated splenocyte feeder cells (a). Alternatively, splenocytes (1×10^5, 2×10^4 and 4×10^3) were stimulated for 7 days with 4 mg CpG-ODN 2006 ml^{-1} with mitomycin-treated feeder cells (b). gp120-specific antibodies were measured in the media by ELISA, and A_{450} readings are shown. Alternatively, splenocytes (1.25×10^6 cells ml^{-1}) were stimulated for 24 h with 1 μg Env peptide pool ml^{-1} at 37 °C, 5 % CO_2 in the presence of 1 μg GolgiPlug (BD Bio Sciences) ml^{-1} for the final 6 h of stimulation. CD8 and CD4 effector memory (CD44^{hi}CD62L$^-$) (c) and central memory (CD44^{hi}CD62L$^+$) (d) T-cell responses, as measured by Th1 cytokine production.

that were boosted by IM/IM, IM/IN and IN/IM regimens, relative to the IN/IN regimen, these differences were not statistically significant.

DISCUSSION

Mucosal immunity is important for protection against pathogens, including HIV-1 (Asahi-Ozaki *et al.*, 2004; Ozawa *et al.*, 2009; Tamura *et al.*, 1988; Watanabe *et al.*, 2002). Early HIV-1 vaccine trials were focused on systemic

immunity and were not designed specifically to elicit mucosal immune responses. Given the failures (Bradac & Dieffenbach, 2009; Robb, 2008; Watkins *et al.*, 2008) and limited successes (Rerks-Ngarm *et al.*, 2009) of HIV-1 vaccines to date, strategies to optimize induction of mucosal anti-HIV-1 immunity (Holmgren & Czerkinsky, 2005; Schoenly & Weiner, 2008) are needed. Approaches that have been explored include immunization via mucosal routes (e.g. IN), targeting antigens to lymph nodes (Finerty *et al.*, 2001; Hinkula *et al.*, 2008; Koopman *et al.*, 2007; Lehner *et al.*, 1999) and administering vaccines with mucosal adjuvants (Connell, 2007; Glenn *et al.*, 2007; Stevceva & Ferrari, 2005).

While DNA prime–protein boost regimens have been evaluated for induction of systemic immunity, little is known about their effect on mucosal responses. We therefore hypothesized that a multimodal DNA prime–protein boost approach using parenteral and mucosal adjuvanted-protein delivery would maximize both mucosal and systemic immunity. To this end, two promising new adjuvants (Advax-M and Advax-P), specifically designed for mucosal and parenteral administration, were tested with gp120 protein. Similar, but unrelated, vaccine strategies have shown induction of long-term HIV-1-specific IgA responses in mucosal secretions and serum when mice were immunized intranasally with DNA encoding gp160 and gp41 peptides (Devito *et al.*, 2000).

Our previous studies have demonstrated the utility of DNA priming prior to protein boost for induction of a strong systemic anti-gp120 antibody response (Cristillo *et al.*, 2006; Pal *et al.*, 2006; Wang *et al.*, 2006, 2008). Those studies used QS-21 adjuvant, which has been found to be potent but is associated with reactogenicity in humans (Kennedy *et al.*, 2008; Petrovsky, 2008). Extending these earlier findings, we now show the importance of the prime–boost regimen for induction of IgA. This was evidenced by the fact that strategies that failed to elicit anti-gp120 IgA responses in vaginal wash (Advax-P1 group) or serum (Advax-P1 group) samples following protein immunization alone induced anti-gp120 IgA following combined prime–boost vaccination (Fig. 2). While parenteral DNA immunization has previously been shown to elicit mucosal and systemic IgA antibodies, responses were weak and/or short-lived (Lai *et al.*, 2007). To enhance such mucosal responses, DNA has been administered mucosally (Bertley *et al.*, 2004; Manrique *et al.*, 2009; Raska *et al.*, 2008; Wang *et al.*, 2004) and/or adjuvants such as GM-CSF (Lai *et al.*, 2007), polyethyleneimine (Huang *et al.*, 2007) or QS-21 (Sasaki *et al.*, 1998) have been used. However, in the current study, naked DNA was administered parenterally followed by adjuvanted-protein boost delivered by mucosal, parenteral or combination routes. This alternate multimodal approach elicited robust and persistent mucosal IgA and systemic humoral and cellular immunity.

The importance of HIV-specific IgA has been demonstrated by studies showing that serum and mucosal IgA from highly exposed, persistently seronegative individuals

inhibited HIV-1 transcytosis (Devito *et al.*, 2000; Mazzoli *et al.*, 1999) and could neutralize primary isolates of many subtypes (Devito *et al.*, 2002). More recently, HIV-specific serum IgA from long-term survivors of HIV infection was shown to neutralize genetically diverse HIV-1 strains (Planque *et al.*, 2010). For IgA characterization in mucosal samples, we did not discriminate between monomeric or secretory (dimeric or multimeric) IgA, nor did we determine whether the origin of IgA was the mucosa or sera. However, given the effectiveness of IgA from both sera and mucosa, such discrimination might not be critical in this instance.

The mechanism(s) by which Advax adjuvants enhanced serum and mucosal IgA levels following DNA priming has yet to be characterized. Other related carbohydrate adjuvants (e.g. γ-inulin) have been shown to enhance B-cell differentiation and antibody production, in part via activation of the alternative complement pathway (Carroll, 1998; Silva *et al.*, 2004). In addition, galactosyl ceramide has been shown to enhance mucosal IgA production via CXCL16–CXCR6-dependent induction of IL-4 by mucosal NKT-cells (Kamijuku *et al.*, 2008). Mucosal adjuvants, such as cholera toxin B subunit, promote IgA via transforming growth factor β1 (Kim *et al.*, 1998) whereas CpG and polyinosinic:polycytidynic acid promote IgA class switching through induction of APRIL (a proliferation-inducing ligand) (Barone *et al.*, 2009; Hardenberg *et al.*, 2007; He *et al.*, 2007; Shang *et al.*, 2008). Future studies will investigate whether Advax adjuvants function via these mechanisms.

Combination mucosal–parenteral delivery strategies using protein alone have been reported to elicit robust mucosal antibody and T-cell responses in mice (Goodsell *et al.*, 2008; Srivastava *et al.*, 2008) and macaques (Barnett *et al.*, 2008). Consistent with these reports, the current study also found induction of strong mucosal immunity following prime–boost immunization (Fig. 4). It is likely that protein immunization via the mucosal route expands DNA-primed B_{mem} and triggers specific homing to mucosal compartments. Subsequent parenteral protein boost further activates B_{mem} in spleen and bone marrow, thereby boosting systemic IgG production. Such distinct compartmentalization of mucosal and systemic immune responses has been described previously (Kantele *et al.*, 1997, 1999; Qadri *et al.*, 1998; Quiding-Järbrink *et al.*, 1997). Future studies will examine whether a coordinated, compartment-driven homing of DNA-primed T- and B-cells occurs following protein immunization in non-human primates.

Involvement of specific IgG subclasses, including IgG2 (Ngo-Giang-Huong *et al.*, 2001) and IgG3 (Scharf *et al.*, 2001), in neutralizing HIV-1 and controlling viraemia have been described. In this study, an adjuvant-dependent broadening of the IgG-subclass response was noted following DNA prime–protein boost immunization that was not seen following administration of DNA or protein alone. It is likely that such broadening of IgG responses by

our vaccine regimen may ultimately provide better control of viraemia in non-human primate efficacy studies.

Persistence of T- and B-cell responses will be of key importance to an effective HIV vaccine, especially for developing countries, where adherence to vaccination schedules may be an issue. In this study, the prime–boost vaccine regimen demonstrated persistent antibody and cell mediated immune (CMI) response over a 23-week period following final immunization. This led to the generation of memory B-cell as well as central and effector memory T-cells (Fig. 6). Recent studies have suggested that effector memory T-cells, which are the predominant memory T-cell population in mucosal compartments, may play a critical role in protection against SIV infection (Hansen *et al.*, 2009). Therefore, such long-lived responses may be important for the containment of HIV-1.

Although a detailed characterization of neutralizing antibodies is difficult to perform in mice, due to limited availability of immune serum, preliminary neutralization assays were performed with pooled sera from each immunized group. While induction of homologous neutralizing antibodies was clearly noted following immunization, titres against live virus ($SHIV_{Ba-L}$) were higher than those against pseudovirus ($HIV-1_{BaL.26}$). This finding may be due, in part, to the sequence heterogeneity amongst these two isolates. Neutralizing titres noted in the IN/IM group were found to be higher than those noted in the IM/IN group. Interestingly, the IN/IM group also demonstrated increased memory B-cell responses compared with the IM/IN group. Such findings, although preliminary, suggest that our vaccine, delivered via the IN/IM route, may have the potential to elicit high-titre neutralizing antibodies and may induce long-term memory B-cell responses. No heterologous neutralization was detected in any of the immunized groups. This finding was not surprising, as the $HIV-1_{Ba-L}$ Env antigen used for this study has been shown previously to elicit type-specific neutralization. The purpose of this selected antigen was primarily to show Env-specific-binding antibody responses in the periphery and in mucosal compartments. Future studies will include Env antigens, based on sequence and/or structural analyses, that yield an immunogen poised to induce broadly neutralizing antibodies.

One area relevant to HIV vaccine development that was not addressed in this study is the efficacy of the candidate vaccine against a defined challenge virus. Specific differences between the mucosal immune systems of rodents and primates limit the use of rodent models for the detailed evaluation of efficacy (Kunisawa *et al.*, 2005; Vajdy & Singh, 2005). While humanized mice models have been developed recently for HIV transmission studies (Denton & Garcia, 2009), the immune responses in these models have not been characterized extensively, thereby limiting their use in vaccine-efficacy studies. Thus, the non-human primate model represents a better alternative for testing the efficacy of this vaccine regimen and is the subject of

ongoing research. Given that neutralization was noted against SHIV$_{Ba-L}$ with the vaccine regimen tested, it is probable that protection against a homologous challenge would be noted in non-human primate studies, as has been observed previously with polyvalent vaccines.

METHODS

Antigens and adjuvants. Codon-optimized HIV-1 *env* gene encoding subtype B (HIV-1$_{Ba-L}$) plasmid DNA and recombinant gp120 vaccine components were prepared as described (Cristillo *et al.*, 2006; Pal *et al.*, 2005, 2006; Wang *et al.*, 2006). Recombinant gp120 was formulated with Advax-M and Advax-P1 adjuvants provided by Vaxine Pty Ltd by simple mixing prior to administration.

Murine immunizations. For the first study, BALB/c mice (5–7 week-old females) were vaccinated with either recombinant gp120 alone or by DNA prime–protein boost immunization. For protein-only immunizations, mice were injected at weeks 0, 2 and 5 with recombinant gp120 (25 µg) either intramuscularly, formulated in Advax-P1 (1 mg) adjuvant, or intranasally, formulated in Advax-M (2 µg) adjuvant. For prime–boost immunizations, mice were immunized intramuscularly with DNA (100 µg) at weeks 0, 2 and 4, and with 25 µg adjuvant-formulated protein at weeks 9 and 11. At 2 weeks post-final immunization, mice were sacrificed and serum, vaginal washes, saliva, faecal pellets and splenocytes were collected to evaluate humoral and cellular immune responses.

For the second study, BALB/c mice (5–7 week-old females) were vaccinated using DNA prime–protein boost combination strategies. Mice were immunized intramuscularly with DNA (100 µg) at weeks 0, 2 and 4, and with adjuvanted gp120 protein (25 µg) at weeks 9 and 11. Alternatively, combination delivery strategies were tested in which protein was administered via the IM route at week 9 and via the IN route at week 11, or vice versa. At 2 and 23 weeks post-final protein immunization, mice were sacrificed and serum, vaginal washes, saliva and splenocytes were collected for analyses.

Peptides. For the murine study, 79 HIV-1 Env (BaL) peptides (15-mers) with 11 overlapping residues were synthesized (Infinity Biotech Research and Resource) that spanned the gp120 Env sequence. These were resuspended in one peptide pool. For T-cell immune assays, cells were stimulated using a final per peptide concentration of 1 µg ml^{-1}.

IFN-γ ELISPOT. The IFN-γ ELISPOT assay was performed using splenocytes and according to the manufacturer's protocol (U-CyTech) as described previously (Cristillo *et al.*, 2006, 2008a; Pal *et al.*, 2005).

CBA. CBA (BD Biosciences) was performed to quantify secreted Th1/Th2 cytokines from splenocytes, as described previously by Cristillo *et al.* (2008a, b).

Measuring gp120-specific IgG responses in serum by ELISA. Sera were assayed for anti-gp120-specific IgG antibodies using an ELISA as described previously by Pal *et al.* (2002). Titres were determined, by ELISA, as the highest dilution of immune serum that produced A_{450} readings greater than or equal to two times the signal detected with a corresponding dilution of pre-immune serum.

Measuring gp120-specific IgG isotype responses in serum. Anti-gp120-specific isotype responses in sera were assayed by ELISA using isotype-specific conjugates. Briefly, recombinant gp120 was coated onto 96-well plates (100 ng per well) (Greiner) and incubated overnight at 4 °C. Plates were washed (PBS pH 7.3/0.01 % Tween)

and blocked [PBS/5 % dried-milk powder (DM)] for 1 h at 37 °C. Diluted (PBS/5 % DM) samples (100 µl) were added to plates and incubated for 1 h at 37 °C. Specific IgG isotypes were detected using rat anti-mouse IgG1–HRP (BD Pharmigen), rat anti-mouse IgG2a–HRP (BD Pharmigen), goat anti-mouse IgG2b–HRP (AbD Serotec) and goat anti-mouse IgG3–HRP (AbD Serotec). Anti-mouse IgG isotype conjugates (diluted 1 : 5000 in PBS/5 % DM) were added to the plates and incubated for 1 h at 37 °C. After washing, 100 µl Enhanced K-Blue tetramethylbenzidine (TMB) substrate (Neogen) was added and the plates were incubated for 15 min at 37 °C. Reactions were stopped with 100 µl 2 M sulfuric acid (LabChem) and A_{450} were measured (SpectraMax Plus 384; Molecular Devices).

Measuring gp120-specific IgA responses in serum and mucosal secretions by ELISA. Saliva and vaginal-wash samples were collected from immunized or control mice as described by Kaminski & VanCott (1999). To rule out blood contamination contributing to transudated serum IgA (Meckelein *et al.*, 2003), all mucosal washes in our study were tested with Haemoccult and shown to be negative. Coating of plates with gp120 and blocking were performed as described above. Samples (100 µl diluted in PBS/5 % DM at a 1 : 5 ratio for saliva and a 1 : 2 ratio for vaginal washes) were added to washed plates and incubated for 1 h at 37 °C. Goat anti-mouse IgA-HRP (100 µl, diluted 1 : 5000 in PBS/5 % DM) (Southern Biotech) was added to plates and incubated for 1 h at 37 °C. Plates were developed with TMB substrate as described above.

Neutralization assay. Neutralization activity was measured with pooled sera (for each group) using a TZM-bl assay where a reduction in luciferase gene expression was measured after a single round of infection in the presence of immune serum compared with untreated control. Viruses were incubated with serial dilutions of duplicate serum samples (50 µl) in complete Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37 °C. Freshly trypsinized TZM-bl cells (10 000 cells in 50 µl complete DMEM medium with 60 µg DEAE–dextran ml^{-1}) were added to each well. For controls, wells received either cells and virus (virus control) or cells alone (background control). After 48 h, 100 µl lysate was transferred to 96-well black plates for measurement of luminescence (Bright-Glo Luciferase assay system; Promega). Neutralization titres are defined as the dilution of serum at which the relative luminescence units (RLU) were reduced by 50 % compared with virus control wells after subtraction of background RLU.

Memory B-cell analysis. At 23 weeks post-final immunization, vaccine-specific memory B-cell responses were evaluated in splenocytes of immunized mice using two murine-adapted stimulation protocols, as described previously (Guan *et al.*, 2009; Slifka & Ahmed, 1996; Traggiai *et al.*, 2004). Splenocytes (1×10^5, 2×10^4 and 4×10^3 cells) were stimulated for 7 days with 4 mg CpG ODN2006 ml^{-1} or ConA supernatant mixture, in the presence of mitomycin-treated splenocyte feeder cells, to activate murine B$_{mem}$ to become antibody-secreting cells as described by Slifka & Ahmed, (1996). Following stimulation, anti-gp120 titres in the supernatants were determined by ELISA (Pal *et al.*, 2002).

Memory T-cell analysis. CD8 and CD4 effector (CD44^{hi}CD62L$^-$) and central (CD44^{hi}CD62L$^+$) memory T-cell responses, measured by intracellular Th1 (TNF-α, IL-2 and IFN-γ) cytokine production, were evaluated in splenocytes of immunized and naive mice at 23 weeks post-final immunization using multi-parameter flow cytometry as described by Cristillo *et al.* (2008b). Splenocytes (1.25×10^6 cells ml^{-1}), stimulated for 24 h with 1 µg Env peptide pool ml^{-1} at 37 °C and 5 % CO_2 (and with 1 µg GolgiPlug ml^{-1} for the final 6 h), were collected, washed (BD FACS wash buffer; BD Biosciences) and stained with anti-murine anti-CD3–APC–Cy7, CD4–PerCP–Cy5.5, CD8–PE–Cy7, CD44–FITC (BD Biosciences)

and CD62L–PE–Texas Red (Invitrogen). Intracellular staining of cells was performed using anti-TNF-α–PE, IFN-γ–PE and IL-2–PE (BD BioSciences). Cells (20 000 each of CD44hi^{+}, CD4^{+} or CD8^{+} memory cells) were acquired using an LSRII cytometer and data were analysed using FACS DIVA (BD BioSciences).

Statistics. A two-tailed Mann–Whitney non-parametric test was performed to assess the statistical significance of the ELISA and CBA data for mice immunized with Advax-M and Advax-P1 in study I. For the ELISA, ELISPOT and CBA data from study II, in which multiple groups of mice were immunized using Advax-M and Advax-P1 by combination routes, the Kruskall–Wallis non-parametric test followed by Dunn's multiple-comparison post-test was used.

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