HIV-1 subtype C Pr55\textsuperscript{gag} virus-like particle vaccine efficiently boosts baboons primed with a matched DNA vaccine

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A DNA vaccine expressing human immunodeficiency virus type 1 (HIV-1) southern African subtype C Gag (pTHGag) and a recombinant baculovirus Pr55\textsuperscript{gag} virus-like particle prepared using a subtype C Pr55\textsuperscript{gag} protein (Gag VLP) was tested in a prime–boost inoculation regimen in Chacma baboons. The response of five baboons to Gag peptides in a gamma interferon (IFN-\gamma) enzyme-linked immunospot (ELISPOT) assay after three pTHGag immunizations ranged from 100 to 515 spot-forming units (s.f.u.) per 10\textsuperscript{6} peripheral blood mononuclear cells (PBMCs), whilst the response of two baboons to the Gag VLP vaccine ranged from 415 to 465 s.f.u. per 10\textsuperscript{6} PBMCs. An increase in the Gag-specific response to a range of 775–3583 s.f.u. per 10\textsuperscript{6} PBMCs was achieved by boosting with Gag VLPs the five baboons that were primed with pTHGag. No improvement in Gag responses was achieved in this prime–boost inoculation regimen by increasing the number of pTHGag inoculations to six. IFN-\gamma responses were mapped to several peptides, some of which have been reported to be targeted by PBMCs from HIV-1 subtype C-infected individuals. Gag VLPs, given as a single-modality regimen, induced a predominantly CD8\textsuperscript{+} T-cell IFN-\gamma response and interleukin-2 was a major cytokine within a mix of predominantly Th1 cytokines produced by a DNA–VLP prime–boost modality. The prime–boost inoculation regimen induced high serum p24 antibody titres in all baboons, which were several fold above that induced by the individual vaccines. Overall, this study demonstrated that these DNA prime/VLP boost vaccine regimens are highly immunogenic in baboons, inducing high-magnitude and broad multifunctional responses, providing support for the development of these products for clinical trials.

INTRODUCTION

The great diversity between human immunodeficiency virus type 1 (HIV-1) subtypes, which are prevalent in various regions of the world, is a major impediment to the development of broad-based prophylactic HIV-1 vaccines. Thus, it may be necessary to develop vaccines that match local epidemics more closely (Morris et al., 2001). In southern Africa, subtype C infections predominate (UNAIDS, 2006).

DNA vaccines encoding HIV or simian immunodeficiency virus (SIV)/simian–human immunodeficiency virus (SHIV) antigens have been studied extensively and shown...
to induce both humoral and cellular immune responses in animal models as well as in humans (Boyer et al., 1997, 2000; Calarota et al., 1998; Estcourt et al., 2004; Giri et al., 2004; Haynes et al., 1994; Letvin et al., 1997; Lu et al., 1995; MacGregor et al., 1998; Yasutomi et al., 1996). However, although DNA vaccines have been shown to be safe, immunization generates low and transient levels of immune responses. Various approaches to augment DNA vaccines have been tested (Barouch et al., 2000; Hemmi et al., 2003; Raviprakash et al., 2003; Xin et al., 1999), including their use in heterologous prime–boost immunization regimens (Allen et al., 2000; Amara et al., 2001, 2005; Boyer et al., 2005; Casimiro et al., 2003a, b; Cher pelis et al., 2001; Hanke et al., 1999; Letvin et al., 1997; Leung et al., 2004; Pal et al., 2006; Robinson et al., 1999; Suh et al., 2006).

HIV-1 Pr55\textsuperscript{gag} precursor protein possesses an ability to self-assemble into non-replicating and non-infectious virus-like particles (VLPs) (Deml et al., 1997; Mergener et al., 1992; Sakuragi et al., 2002; Wagner et al., 1994, 1996a) and elicits strong humoral and cellular immune responses in animals (Deml et al., 1997, 2004; Jaffray et al., 2004; Wagner et al., 1996b), including non-human primates (NHPs) (Montefiori et al., 2001; Paliard et al., 2000; Wagner et al., 1996b). In addition, HIV-1 Pr55\textsuperscript{gag} VLPs are safe, easy to produce and have the potential of including chimaeric antigens (Doan et al., 2005). Their particulate nature and size, which approximates that of HIV-1, make them more likely to stimulate the immune system better than non-particulate antigens. For these reasons, it is justifiable to use Pr55\textsuperscript{gag} VLPs as a booster vaccine in heterologous prime–boost modalities.

Baboons have been used to test the safety and immunogenicity of various candidate human vaccines (Murthy et al., 2006), including HIV vaccines (Casimiro et al., 2003a; Leung et al., 2004; Locher et al., 2004). Although macaques are commonly used as vaccine immunogenicity models, several factors make vaccine studies more attractive in baboons. Importantly there is a high sequence homology between the components of the human immune system and that of the baboon, with the baboon having four subclasses of immunoglobulin analogous to humans, unlike the macaque, which has only three subclasses (Damian et al., 1971; Martin, 1982). Safety issues also favour the use of baboons over macaques. Unlike macaques, baboons cannot be infected with cercopithecine herpesvirus 1 (monkey B virus), a potentially fatal health hazard to animal handlers and care takers (Huff & Barry, 2003; Whitley & Hilliard, 2001).

Previous studies by our group have reported the construction of a gag DNA (pTHGag) (van Harmelen et al., 2003) vaccine expressing the gag gene from a South African HIV-1 subtype C primary isolate, Du422, and an HIV-1 subtype C Pr55\textsuperscript{gag} VLP (Gag VLP) vaccine using Du422 Gag protein (Williamson et al., 2003). pTHGag was shown in the mouse model to induce a potent, long-lived cytotoxic lymphocyte response (van Harmelen et al., 2003) and efficiently primed for Gag VLPs in a prime–boost regimen (Jaffray et al., 2004). There are no studies in NHPs testing VLPs as a heterologous boost. In the present study, we investigated the immune response of baboons to these two vaccines and the ability of Gag VLPs to boost the immune system of Chacma baboons after priming three or six times with pTHGag. Both vaccines induced low Gag-specific cellular and humoral immune responses. Gag VLPs boosted the immune response to pTHGag to a high magnitude, irrespective of whether the immune system was primed three or six times by pTHGag.

**METHODS**

**Vaccine preparation.** The vaccine construct pTHGag expressing the HIV-1 subtype C Gag gene of Du422 has been described previously (van Harmelen et al., 2003; Williamson et al., 2003). The vaccine stock for baboon inoculations was manufactured by Aldevron and resuspended at 2 mg ml\textsuperscript{-1} in PBS and stored at −80 °C.

Gag VLPs were produced using a Bac-to-Bac Expression System (Invitrogen) and recombinant baculovirus as described previously (Jaffray et al., 2004). Gag content and integrity were evaluated by Western blotting and the purified Gag VLPs were screened to ensure that they were free from microbial contamination and that the endotoxin level was below 1.0 EU ml\textsuperscript{-1}. The inoculum stocks, with a Gag concentration of 118 μg ml\textsuperscript{-1}, were stored at −80 °C.

**Baboons and immunization.** Wild-caught Chacma baboons, housed at the South African Medical Research Council’s animal facility in Cape Town, South Africa, were used in these experiments. Eleven healthy baboons, weighing between 6 and 15 kg and serologically negative for SIV antibodies, were randomly grouped (van Harmelen et al., 2003; Whitley & Hilliard, 2001). The schedule for immunization of baboons in study 1 and study 2 with pTHGag and Gag VLPs is indicated in Results. Both pTHGag (5 mg per dose) and Gag VLP (29 μg per dose) were inoculated intramuscularly in the quadriceps muscles using a hypodermic needle. Blood samples for serum and peripheral blood mononuclear cells (PBMCs) were obtained at pre-immunization and various times post-immunization. Animals were monitored for local reactions at the inoculation sites. Ketamine hydrochloride was used to anaesthetize the baboons for all procedures. This study was reviewed and approved by the Animal Ethics Committee of the University of Cape Town.

**Isolation of baboon PBMCs.** PBMCs were isolated from heparinized blood using Ficoll gradient centrifugation and then resuspended at 1 × 10\textsuperscript{7}–2 × 10\textsuperscript{7} PBMCs ml\textsuperscript{-1} in fetal bovine serum (FBS; Gibco) containing 10% DMSO (Sigma) and cryopreserved.

**Preparation of Gag peptide pools.** Gag-specific T cells in baboon PBMCs were detected using a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assay with 66 synthetic peptides spanning the entire HIV-1 subtype C Gag protein sequence. These peptides, based on the subtype C Gag consensus sequence, were 15–18 aa in length and overlapped by 10 aa. The peptides were dissolved in 100% DMSO (Sigma) at a concentration of 10 mg ml\textsuperscript{-1} and used to make two sets of peptide pools. One set contained the five major peptide pools, P1–P5, with 14 overlapping consecutive peptides in...
each pool. The other set contained 14 matrix peptide pools, M1–M14, with five non-overlapping peptides in each pool.

**IFN-γ ELISPOT assay.** PBMCs were incubated in triplicate at 2 × 10^5 cells per well with peptide pools P1–P5 at a final concentration of 4 μg ml^{-1} for each peptide. Triplicate wells with cells and R10 medium (RPMI 1640 containing 10% FBS; Gibco) only served as negative controls to determine background responses. The IFN-γ ELISPOT assay was conducted as described previously (Chege et al., 2005; Mashishi & Gray, 2002) using ELISPOT plates (MultiScreen-IP; Millipore) coated with purified anti-human IFN-γ clone 1-D1K (Mabtech) and the anti-human IFN-γ clone 7-B6-1 (Mabtech) to detect cells secreting IFN-γ. An ImmunoSpot (Cellular Technology) automated plate analyser together with ImmunoSpot version 3.2 software was used to scan and count the spots in each well. The mean number of spots in triplicate wells that did not differ by more than 10 spots was normalized to spots per 10^6 PBMCs to give the IFN-γ spot-forming units (s.f.u.) per 10^6 PBMCs. A response to any peptide pool that was equal to or greater than the peptide pool cut-off value of 45 s.f.u. per 10^6 PBMCs for baboons in study 1 and 20 s.f.u. per 10^6 PBMCs for baboons in study 2 was considered positive. This value was the mean response of baboon PBMCs before immunization to the five major peptide pools plus 4 SD. The cumulative PBMC response to Gag was calculated as the sum of the s.f.u. per 10^6 PBMCs for each of the five major peptide pools.

The individual peptides to which PBMCs were responding were identified using the five major peptide pools (P1–P5) and the 14 matrix peptide pools (M1–M14) as stimuli in the IFN-γ ELISPOT assay. The use of these peptides in this ‘pool–matrix’ format allowed responses to any one of the major peptide pools to be cross-referenced to a response in one of the matrix pools (Anthony & Lehmann, 2003; Masemola et al., 2004a), thus predicting an individual peptide response. Confirmation of the response to the identified peptide was carried out in a further assay and the cut-off values established earlier were used for determination of a positive response.

**Intracellular IFN-γ staining.** PBMCs (2 × 10^6) in 100 μl R10 medium were stimulated with a single Gag peptide (4 μg ml^{-1}) in the presence of low-endotoxin (<0.01 μg ml^{-1}), azide-free monoclonal antibodies to human CD28 (clone CD28.2; BD Pharmingen) and CD49d (clone 9F10; BD Pharmingen), each at a final concentration of 1 μg ml^{-1}. Control reactions did not contain peptide. Cells were incubated at 37 °C for 2 h at which time 900 μl R10 medium containing 10 μg brefeldin A was added and the incubation was continued for a further 14 h. The cells were stained with mouse anti-monkey CD3–fluorescein isothiocyanate (FITC) (clone FN-18; BioSource), anti-human CD4–allophycocyanin (APC) (clone SK3; BD), anti-human CD8–APC (clone SK1; BD) or corresponding isotype controls. The cells were permeabilized and then stained for intracellular IFN-γ using mouse anti-human IFN-γ–recombinant phycoerythrin (PE) (clone 4S.B3; BD Pharmingen) or the corresponding isotype control. Cells were washed and resuspended in fixing solution (CellFix; BD) and then acquired using a BD FACSCalibur flow cytometer and analysed using CellQuest (BD) software.

**Detection of released cytokines by cytometric bead array (CBA) assay.** PBMCs (2 × 10^6 ml^{-1}) were incubated for 16 h in R10 medium containing individual Gag peptides (4 μg ml^{-1}) or R10 medium only (background responses). Low-endotoxin (<0.01 μg ml^{-1}), azide-free monoclonal antibodies to human CD28 (clone CD28.2; BD Pharmingen) and CD49d (clone 9F10; BD Pharmingen) were each added at a final concentration of 1 μg ml^{-1}. The culture supernatants were harvested and stored frozen at −80 °C until the concentration of cytokines in the culture supernatants was determined using a CBA NHP Th1/Th2 cytokine kit (BD), which quantifies IFN-γ, tumour necrosis factor (TNF-α), interleukin (IL)-2, IL-4, IL-5 and IL-6. Data are presented as the net amount of cytokine (pg ml^{-1}) released in the presence of the Gag peptide, after the negative response in the absence of peptide had been subtracted. The mean background responses varied from 1.9 to 22.5 pg ml^{-1}. The cut-off values for a positive response (mean background response plus 1 SD) in pg ml^{-1} were as follows: IFN-γ: 9.4; TNF-α: 33.0; IL-2: 17.6; IL-4: 5.5; IL-5: 2.3 and IL-6: 5.2.

**Anti-HIV-1 p24 antibody assay by ELISA.** Maxisorp plates (Nalge Nunc) were coated with 0.1 μg HIV-1 recombinant p24 protein (Quality Biological) per well. Sera obtained at various time points were added to duplicate wells at a dilution of 1:40, whilst those from six key time points were also used in 11 twofold serial serum dilutions, starting at 1:40. A negative-control serum, consisting of a pool of pre-immune baboon sera at 1:40 dilution, was included in each microtitre plate in quadruplicate. Serum IgG was detected using rabbit anti-monkey IgG horseradish peroxidase (Sigma) diluted 1:2000 and developed with tetramethylbenzidine (Kirkgaard & Perry Laboratories). The results are presented as an absorbance value or end-point antibody titre, which was defined as the reciprocal of the highest serum dilution with an absorbance that was equal to or greater than the mean absorbance of the negative control serum plus 2 SD.

**Anti-Gag antibody assay by Western blotting.** The presence of Gag antibodies in baboon sera was confirmed using a New LAV Blot 1 kit (Bio-Rad Laboratories) following the manufacturer’s instructions, but with the anti-human IgG alkaline phosphatase conjugate solution substituted with anti-monkey IgG conjugated to alkaline phosphatase (Sigma) and used at a 1:500 dilution.

**RESULTS**

**Local reactions at injection sites**

Inoculation of baboons in study 1 and study 2 with pTHGag and Gag VLPs, either individually or in the prime–boost regimen, did not induce any noticeable local reaction at the injection site and all of the baboons maintained good health throughout the study period.

**Responses to Gag in the IFN-γ ELISPOT assay**

Four of the five baboons in group A (study 1), which were primed with three inoculations of pTHGag each 4 weeks apart, developed low IFN-γ responses to Gag, detectable only after the third DNA inoculation (Fig. 1a). Peak cumulative responses to the Gag peptide pools for the individual baboons were variable, ranging from 100 to 515 s.f.u. per 10^6 PBMCs. Two baboons (660 and 663) reacted to all five Gag peptide pools, whereas the other two baboons (592 and 369) recognized two or three Gag peptide pools. These responses for all four baboons declined over time (Fig. 1a).

Baboons in group A (study 1) were given a Gag VLP booster inoculation 43 weeks after the last pTHGag inoculation. All five animals developed strong responses to Gag, with peak cumulative responses occurring 2–4 weeks after the Gag VLP inoculation and ranging from 775 to 3583 s.f.u. per 10^6 PBMCs in the IFN-γ ELISPOT assay (Fig. 1a). For four baboons, these responses were directed...
to all five Gag peptide pools, whilst one baboon (592) reacted to four Gag peptide pools. With time, these responses to the Gag peptide pools declined, and by week 63 the range of responses in the IFN-γ ELISPOT assay varied from 222 to 616 s.f.u. per 10^6 PBMCs. A second Gag VLP inoculation given at week 63 induced a response of smaller magnitude than that produced by the initial Gag VLP inoculation, except for baboon 592 where the response was restored. By week 71, the response had declined considerably, with the cumulative response ranging from 0 to 250 s.f.u. per 10^6 PBMCs and directed to one or two Gag peptide pools (Fig. 1a).

The two baboons in group B (study 1), vaccinated with Gag VLPs only, developed a Gag-specific response detectable 2 weeks after the inoculation, with the cumulative responses to the Gag peptide pools ranging from 415 to 465 s.f.u. per 10^6 PBMCs (Fig. 1b). For baboon 664, this response was directed to three Gag peptide pools, whilst Baboon 674 directed the response to all five Gag peptide pools. A booster Gag VLP injection given at week 63 induced a response in both animals but did not boost the initial response. The cumulative responses to Gag peptide pools at the peak after boost ranged from 207 to 219 s.f.u. per 10^6 PBMCs and were predominantly directed to Gag peptide pool 3 (Fig. 1b).

As the immune response to Gag for the baboons in study 1 was low after three pTHGag immunizations, we investigated in a second study (study 2) whether more immunizations with pTHGag would increase the magnitude of the immune response. Two baboons (348 and 500; group C) were given four inoculations of pTHGag each 4 weeks apart as for study 1, followed by further pTHGag immunizations at weeks 34 and 56 when it was expected that memory responses from the initial immunizations would be effective (Fig. 1c). Responses to Gag were just above background levels after the fourth DNA vaccination. After the fifth DNA immunization, the cumulative response peaked at 91 and 273 s.f.u. per 10^6 PBMCs for baboons 348 and 500, respectively. At 2 weeks after the sixth pTHGag inoculation, a cumulative response of 130 and 280 s.f.u. per 10^6 PBMCs was detected for baboons 348 and 500, respectively. These responses were to three or four Gag peptide pools (Fig. 1c). A Gag VLP inoculation given at week 75 induced responses to Gag peptides in all five Gag peptide pools. For baboon 348, a peak cumulative response of 2254 s.f.u. per 10^6 PBMCs measured 2 weeks after the VLP boost was 17-fold above the peak Gag-specific response induced by pTHGag. Baboon 500 reached a peak cumulative response to Gag of 1262 s.f.u. per 10^6 PBMCs at 4 weeks after the Gag VLP injection, which was fourfold above the peak Gag-specific response induced by pTHGag. However, these responses declined with time in a similar manner to that observed in study 1. To determine whether long-term memory cells that could respond to further antigen stimulation were induced by the prime–boost inoculation regimen, baboon 348 was monitored at week 127, a year after the VLP booster inoculation. A low Gag response to three Gag peptide pools (187 s.f.u. per 10^6 PBMCs) was detected. A Gag VLP boost given at week 164 increased the cumulative response approximately twofold (460 s.f.u. per 10^6 PBMCs) (Fig. 1c).

The two baboons in group D (baboons 357 and 499; study 2) received two Gag VLP inoculations, 12 weeks apart. Following the first Gag VLP inoculation, a Gag-specific response, which was predominantly to Gag peptide pool 3, was detected (Fig. 1d). The magnitude and kinetics of this response varied widely, reaching a peak of 854 and 246 s.f.u. per 10^6 PBMCs for baboons 357 and 499, respectively, at 4 and 8 weeks after the Gag VLP inoculation. A second Gag VLP inoculation induced a peak response of 943 and 327 s.f.u. per 10^6 PBMCs for baboons 357 and 499, respectively. These responses were directed to Gag peptide pool 3, similar to the response observed after the first Gag VLP inoculation. Further observation of baboon 357 at week 127 showed a response just above background level (62 s.f.u. per 10^6 PBMCs). A third boost with Gag VLPs at this time increased the magnitude to 400 s.f.u. per 10^6 PBMCs (Fig. 1d).

**Individual Gag peptide responses**

The individual peptides in the five Gag peptide pools to which the PBMCs isolated at week 53 (study 1, 2 weeks after the first Gag VLP injection) and at week 79 (study 2, 4 weeks after the first Gag VLP injection) were responding were identified using a ‘pool–matrix’ strategy in the IFN-γ ELISPOT assay as described in Methods. PBMCs from baboons receiving the prime–boost regimen targeted between two and 13 Gag peptides distributed across the three regions of the Gag protein (Table 1). In contrast, PBMCs from baboons receiving Gag VLPs alone targeted two or three Gag peptides, which were restricted to the p24 region. The sequences of the identified peptides and those known to be recognized by PBMCs from people with early HIV-1 subtype C infection (Masemola et al., 2004a, b) are also indicated in Table 1.

**Phenotype of Gag-specific PBMCs**

The phenotype of the lymphocytes producing Gag-specific IFN-γ 2 weeks (study 1) or 4 weeks (study 2) after a VLP vaccination was investigated (Fig. 2 and Table 2). PBMCs were stimulated with Gag peptide pool 3 or an individual peptide found to be reactive in the IFN-γ ELISPOT assay (Table 1) and responding cells were detected by intracellular IFN-γ staining. Some peptides appeared to contain both CD4^+ and CD8^+ T-cell epitopes (Fig. 2 and Table 2).

**Spectrum of cytokines**

The spectrum of cytokines produced by PBMCs isolated from three baboons in study 1, group A (369, 660 and 663) at week 53, 2 weeks after the Gag VLP boost, was
(a) Group A (study 1)

(b) Group B (study 1)
measured. PBMCs were cultured for 16 h in the presence of an individual peptide identified to be responsive in the IFN-γ ELISPOT assay (Table 1) and the culture supernatants were assayed for Th1 and Th2 cytokines. For all three baboons, IL-2 and TNF-α were the major components of the supernatant (Fig. 3). Low levels of IFN-γ were only detected in baboon 633. The IL-4, IL-5 and IL-6 content of the supernatant was very low.

HIV-1 Gag antibody responses

Three immunizations with pTHGag induced low anti-p24 antibody levels in four out of five baboons in group A (study 1), which peaked at week 12, 4 weeks after the third pTHGag inoculation (Fig. 4a and b, left panels). Comparable anti-p24 antibody levels were detected for group C baboons (study 2), which were vaccinated with six pTHGag injections (data not shown).

No anti-p24 antibodies could be detected in the sera of the two baboons in group B or D after receiving a single Gag VLP vaccination. However, anti-p24 antibodies were induced in these baboons by a Gag VLP booster inoculation. The antibody titres for baboons in both groups ranged from 160 to 1280 (Fig. 4a and b, right panels; data not shown for group D). These antibody levels for the two groups of baboons decreased slowly with time.

All five baboons in group A (study 1), primed with pTHGag and then boosted with Gag VLPs at week 51, 43 weeks after the last pTHGag prime, developed high
Table 1. Gag peptides targeted by baboon PBMCs, as identified by a pool–matrix IFN-γ ELISPOT mapping strategy

PBMCs obtained from baboons in study 1 at 2 weeks after the first Gag VLP inoculation and in study 2 at 4 weeks after the first Gag VLP inoculation were used in a pool–matrix IFN-γ ELISPOT mapping strategy (see Methods) to predict peptides to which the cells were responding. The confirmed response to the identified peptide, above the cut-off value of 45 (study 1) or 20 (study 2) IFN-γ s.f.u. per 10⁶ PBMCs, is given as the net IFN-γ s.f.u. per 10⁶ PBMCs. Peptide sequences shown in italics indicate those reported as being targeted by PBMCs from individuals with early HIV-1 subtype C infection (Masemola et al., 2004a, b). Underlined amino acid sequences show an overlap with those of an adjacent Gag peptide that is targeted by the same baboon PBMCs, indicating the possibility of the Gag peptides sharing the same cytotoxic lymphocyte epitope.

<table>
<thead>
<tr>
<th>Inoculation regimen</th>
<th>Baboon number</th>
<th>Gag peptides identified after Gag VLP inoculation</th>
<th>Response (IFN-γ s.f.u. per 10⁶ PBMCs)</th>
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<tbody>
<tr>
<td>pTH Gag/Gag VLPs</td>
<td>369 p24 P3</td>
<td>40 GPKEPFRDYVDRFDKTLR 243</td>
<td></td>
</tr>
<tr>
<td>pTH Gag/Gag VLPs</td>
<td>592 p17 P1</td>
<td>4 GKKHYMLKLHVLVWASREL 46</td>
<td></td>
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<tr>
<td>pTH Gag/Gag VLPs</td>
<td>p24 P2</td>
<td>24 PMFTALSEGATPQDLNTM 238</td>
<td></td>
</tr>
<tr>
<td>Gag VLPs alone</td>
<td>624 p17 P1</td>
<td>5 KHLVWASRELPAL 68</td>
<td></td>
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<tr>
<td></td>
<td>p24 P3</td>
<td>36 PVGDHYWKRIWIIGLNKIV 238</td>
<td></td>
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<tr>
<td></td>
<td>p2p7p1p6 P4</td>
<td>46 TILRALGPGASLEEMMTAT 238</td>
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<tr>
<td></td>
<td>65 PKQEPKDRPLTSLK 63</td>
<td></td>
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<tr>
<td>Gag VLPs alone</td>
<td>660 p17 P1</td>
<td>6 ASRELFALNPGLL 358</td>
<td></td>
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<td></td>
<td>p24 P4</td>
<td>34 STLQEIAWMTSPPPV 121</td>
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<td>Gag VLPs alone</td>
<td>663 p17 P1</td>
<td>64 RFEETTPAPQKEFKDREPL 396</td>
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<tr>
<td></td>
<td>p24 P3</td>
<td>36 GKKHYMLKLHVLVWASREL 251</td>
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<td>51 QANSAIMQRSNFKGPKR 103</td>
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<td>Gag VLPs alone</td>
<td>664 p24 P3</td>
<td>41 YVDFRKTLDRLAQATQDV 1076</td>
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<tr>
<td></td>
<td>674 p24 P3</td>
<td>36 YVDFRKTLDRLAQATQDV 1076</td>
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anti-p24 levels by week 53 (Fig. 4a and b, left panels) The range of anti-p24 titres for these five baboons was 640–10 240. By week 63, the titres had decreased to a range of 320–2560. A boost with Gag VLPs at week 63 led to an increase in titres, which ranged from 1280 to 20 480, followed by a decline over time. Similarly, Gag VLPs given to the two baboons in group C (study 2), after six pTHGag vaccinations, boosted the anti-p24 levels to titres of 1280 and 40 960 for baboons 348 and 500, respectively, followed by a decline over time (data not shown).

Confirmation that the antibodies measured in the ELISA assay were anti-p24 antibodies was obtained using a commercial Western blot system (Fig. 4c). Sera at week 65 from baboons in group A (baboons 592, 624, 660 and 663) that had been primed with pTHGag and then boosted with Gag VLPs were used in the assay. Bands corresponding to Gag proteins p18, p24/25, p40 and p55 were detected on the Western blot strips (Fig. 4c, lanes 2). No bands were detected with pre-inoculation sera (Fig. 4c; lanes 1).

### DISCUSSION

In this study, we evaluated our HIV-1 subtype C vaccines, pTHGag and Gag VLPs, in baboons. These vaccines have previously been shown to be immunogenic in mice (Jaffray et al., 2004; van Harmelen et al., 2003). The baboons had no adverse effects to these vaccines. They developed low Gag-specific cellular and antibody responses to the individual DNA and VLP vaccines. The magnitude of responses to the DNA vaccinations could not be improved by increasing the number of vaccinations. Baboons given four DNA vaccine vaccinations at intervals of 4 weeks followed by two further vaccinations at intervals of around 20 weeks did not show improved Gag cellular and antibody responses compared with those baboons that received just three DNA vaccine injections at 4-weekly intervals. This may indicate that, after multiple injections, the DNA vaccine induces very low frequencies of memory cells that respond at a level that is too low to detect in the IFN-γ ELISPOT. Although responses to the Gag VLPs were low, they could be detected by 2 weeks after the vaccination and long-lived IFN-γ ELISPOT responses were induced.

Irrespective of the number of DNA vaccine injections used in this study to prime the baboon immune system, the Gag-specific cellular immune and antibody responses induced by the vaccine were sufficient for an effective boost with a Gag VLP vaccine. High antibody titres and a high magnitude of Gag-specific IFN-γ-producing cells were detected in all baboons that received the DNA prime/Gag

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**Table 1. cont.**

<table>
<thead>
<tr>
<th>Inoculation regimen</th>
<th>Baboon number</th>
<th>Gag peptides identified after Gag VLP inoculation</th>
<th>Response (IFN-γ s.f.u. per 10^6 PBMCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gag region Peptide pool Peptide number Amino acid sequence</td>
<td></td>
</tr>
<tr>
<td>(b) Study 2</td>
<td>pTH Gag/Gag VLPs</td>
<td>348 p17</td>
<td>P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>348 p24</td>
<td>P2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>348 p3</td>
<td>P3</td>
</tr>
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<td></td>
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<td>348 p3</td>
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<td></td>
<td></td>
<td>348 p4</td>
<td>P4</td>
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<td></td>
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<td>624 p24</td>
<td>P2</td>
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<td>P5</td>
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<tr>
<td></td>
<td></td>
<td>624 p5</td>
<td>P5</td>
</tr>
<tr>
<td></td>
<td>pTH vector/Gag VLPs</td>
<td>357 p24</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>499 p24</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>499 p3</td>
<td>P3</td>
</tr>
</tbody>
</table>

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VLP boost inoculation regimen. The cellular response after the prime and boost was broad, with cells responding to peptides in all regions of Gag. In addition, the prime–boost inoculation regimen appeared to induce a long-term cellular response in some animals, which was detectable up to 89 weeks after the Gag VLP immunization. However, further boosting with Gag VLPs suggested that only maintenance of the magnitude of the cellular response occurred, whilst the humoral response was boosted.

Subtype C vaccines have not been tested in baboons, but there are two reports using baboons to test the immunogenicity of HIV-1 subtype B vaccines (Casimiro et al., 2003a; Leung et al., 2004). These studies indicated that a Gag DNA vaccine had to be administered with adjuvant to prime the immune response of baboons to a fivefold boost of the immune response with either adenovirus serotype 5 (Ad5) expressing Gag or modified vaccinia virus Ankara (MVA) expressing Gag. When the DNA prime was with a vaccine expressing both Gag and Env, multiple inoculations induced low antibody levels and lymphocyte proliferative responses, which were boosted efficiently with Gag VLPs (Leung et al., 2004). In contrast to these studies, we showed that our baboons responded to a DNA vaccine without the need for adjuvant and that the response was sufficient to prime the immune system to a boost with a Gag VLP vaccine.

Macques are the NHP used most often to test the immunogenicity of HIV-1 vaccines. The level of response of macaques to DNA vaccines is generally very low, as we found for our baboons, but acts as a sufficient prime for the immune system for a boost to recombinant MVA and Ad5 vaccines expressing identical proteins to the DNA vaccine (Allen et al., 2000; Amara et al., 2001; Casimiro et al., 2003b; Hanke et al., 1999; Suh et al., 2006). However, other studies indicate that higher primary immune responses to a DNA vaccine can be achieved if the expressed protein forms a VLP in vitro (Ellenberger et al., 2005; Montefiori et al., 2001; Smith et al., 2004). Baculovirus-produced Pr55gag VLPs or chimaeric VLPs with glycoprotein on the outer surface of the VLP can induce Gag-specific T cells with cytolytic activity and antibodies in macaques (Notka et al., 1999; Paliard et al., 2000; Wagner et al., 1998). Low Gag-specific responses, detected in macaques after vaccination with purified Gag-Pol-Env VLPs and adjuvant, were greatly enhanced if these VLPs were co-injected with a DNA vaccine expressing the pseudovirion and adjuvant (Montefiori et al., 2001; Paliard et al., 2000; Wagner et al., 1998).

Table 2. Phenotype of baboon PBMCs responding to individual Gag peptides

Cryopreserved baboon PBMCs obtained 2 weeks after the first Gag VLP inoculation (study 1) or 4 weeks after the first Gag VLP injection (study 2) were incubated for 16 h with selected Gag peptides followed by lymphocyte phenotyping and intracellular IFN-γ staining. Data in bold indicate positive responses above background in the absence of peptide. The mean background frequency was 0.14 ± 0.07% (n=11). The amino acid sequence of the individual peptides used in the assay is indicated in Table 1.

<table>
<thead>
<tr>
<th>Inoculation regimen</th>
<th>Baboon number</th>
<th>Gag peptide(s)</th>
<th>CD3+CD4+IFNγ+ (%)</th>
<th>CD3+CD8+IFNγ+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTHGag/Gag VLPs (study 1, group A)</td>
<td>369</td>
<td>Peptide 41</td>
<td>0.15</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>369</td>
<td>Pool 3</td>
<td>0.35</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>592</td>
<td>Peptide 51</td>
<td>0.25</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>624</td>
<td>Peptide 36</td>
<td>0.35</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>Peptide 37</td>
<td>0.16</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>663</td>
<td>Peptide 41</td>
<td>0.16</td>
<td>0.25</td>
</tr>
<tr>
<td>Gag VLPs (study 1, group B)</td>
<td>664</td>
<td>Peptide 40</td>
<td>0.10</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>674</td>
<td>Peptide 41</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>663</td>
<td>Peptide 41</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>pTHGag/Gag VLPs (study 2, group C)</td>
<td>348</td>
<td>Peptide 6</td>
<td>0.34</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>Peptide 18</td>
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<td>1.02</td>
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<td></td>
<td>348</td>
<td>Peptide 40</td>
<td>1.37</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>Peptide 38</td>
<td>0.16</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>Peptide 51</td>
<td>0.25</td>
<td>0.21</td>
</tr>
<tr>
<td>pTH/Gag VLPs (study 2, group D)</td>
<td>357</td>
<td>Peptide 40</td>
<td>0.07</td>
<td>0.57</td>
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<tr>
<td></td>
<td>357</td>
<td>Peptide 41</td>
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<td>0.37</td>
</tr>
<tr>
<td></td>
<td>357</td>
<td>Pool 3</td>
<td>0.05</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>499</td>
<td>Peptide 36</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>499</td>
<td>Pool 3</td>
<td>0.37</td>
<td>0.18</td>
</tr>
</tbody>
</table>
We chose instead the baboon model to test our HIV vaccines. Baboons are abundant in southern African and are readily available, which makes them the NHP of choice for testing our HIV-1 subtype C vaccines prior to clinical trial in humans. They are wild-caught and outbred, with diverse genetic backgrounds between animals, suggesting that immune responses to specific immunogens delivered by vaccines may reflect more accurately what would occur when testing these vaccines in humans.

As we wanted to use the baboon as our NHP model, we needed to establish that immunogenicity data generated using the baboon model is as valuable as that obtained from studies using macaques. This would help with making the decision whether or not to proceed to clinical trials based on the immunogenicity data from baboons. Results in two reports by Casimiro et al. (2003a, b) in which rhesus macaques and baboons were immunized in two separate experiments with the same DNA and Ad5 HIV-1 gag vaccines gave similar total responses in an IFN-γ ELISPOT assay, suggesting that either baboons or macaques can be used.

An important finding in this study was that the recall response of baboon PBMCs after the Gag VLP boost was to peptides that contained epitopes recognized by HIV-1 subtype C-infected individuals (Masemola et al., 2004a, b).
Thus, Du422, the HIV-1 isolate from which this vaccine was derived, is relevant for designing vaccines against subtype C HIV-1 infection. The phenotype of the Gag-specific cells induced by the vaccination regimen and the spectrum of cytokines released in response to a specific peptide is predictive of possible vaccine-induced efficacy.

The role that HIV-1 CD8\(^+\) and CD4\(^+\) T cells play in protection after challenge is well accepted. SIV Gag-Pol-Env-specific CD8\(^+\) and CD4\(^+\) T cells induced by an SIV Gag-Pol-Env vaccine given as a DNA prime/recombinant MVA boost vaccination regimen to macaques was shown to control a challenge with the virulent SHIV strain 89.6P virus (Amara et al., 2001; Sadagopal et al., 2005; Smith et al., 2004). The importance of vaccine-specific CD4\(^+\) T cells appears to be in their ability to preserve memory CD4\(^+\) T cells, which is surmised to be associated with protection afforded to macaques primed with plasmid DNA and boosted with a recombinant Ad5, both encoding SIV proteins (Edwards et al., 2002; Letvin et al., 2006; Mattapallil et al., 2006). The IFN-\(\gamma\) ELISPOT results obtained from the present study were unable to show whether the cellular immune response was from that of responding CD4\(^+\) and/or CD8\(^+\) T cells. The phenotype of the lymphocytes responding with relatively high frequency to an individual peptide in the IFN-\(\gamma\) ELISPOT assay was
investigated. Some of these individual peptides appeared to contain both CD4⁺ and CD8⁺ epitopes. CD8⁺ T cells with cytolytic function have been induced in macaques vaccinated with HIV-1 VLPs (Notka et al., 1999; Paliard et al., 2000; Wagner et al., 1998). The Gag-specific CD4⁺ T cells induced by our vaccination regimen would be important for the development of Gag-specific CD8⁺ T cells with cytotoxic function, as well as the observed development of HIV-1 p24 antibodies measurable after a pTHGag prime and Gag VLP boost. Although the value of binding antibodies is not clear, it can be proposed that they are important for preventing infection of bystander CD4⁺ T cells.

Virus control in the macaque is also associated with the ability of HIV-specific CD4⁺ and CD8⁺ T cells to produce both IL-2 and IFN-γ (Mooij et al., 2004). Rhesus macaques that developed strong IFN-γ, IL-2 and IL-4 responses after immunization with a vaccine containing a Tat-Env-Gag combination were protected from disease progression after SHIV 89.6P challenge. Also, it was observed that each individual antigen induced a different cytokine profile. Immunization with Env or Tat induced mainly a Th2-type response with marked IL-4 and IL-2 responses but low or absent IFN-γ, whereas immunization with Gag induced a Th1-type profile dominated by IFN-γ and IL-2 responses and lower IL-4 production (Mooij et al., 2004). In the present study, the spectrum of cytokines released during stimulation of baboon PBMCs, taken after a prime with pTHGag and a boost with Gag VLPs, with a peptide to which a high response was obtained in the IFN-γ ELISPOT assay indicates that IFN-γ production may be a minimal component, whilst IL-2 is a major component of the total cytokine mix released during peptide stimulation. The phenotype of the cells producing the measured cytokines of cytolesis was not investigated and may well be CD8⁺ when the phenotype of the cell responding to the peptide in the intracellular cytokine assay is considered. However, CD4⁺ T cells may also be involved. The level of IL-2 being produced by Gag-specific baboon PBMCs after the pTHGag prime and Gag VLP boost in this study may have encouraging outcomes for the protective function of the vaccine-induced cells.

Recent detailed studies of immune responses to HIV-1 infection show that a Gag-specific response is crucial for an effective cell-mediated immune response, supporting the thinking that HIV-1 Gag is an essential component for an HIV-1 vaccine to be effective (Edwards et al., 2002; Kiepiela et al., 2007; Ramduth et al., 2005). These studies demonstrated that Gag-specific responses were the dominant CD4⁺ T-cell response to HIV (Ramduth et al., 2005), whilst the breadth of Gag-specific responses and preferential targeting of Gag by CD8⁺ T cells was associated with lower viremia (Kiepiela et al., 2007; Ramduth et al., 2005). The low viral loads and control of infection by CD8⁺ T cells were unrelated to HIV epitope sequence conservation and HLA specificity (Kiepiela et al., 2007). However, multigene vaccines in the macaque model have been shown to be valuable for enhanced protection (Doria-Rose et al., 2003).

Overall, the present study demonstrated that the Chacma baboon is valuable for testing the immunogenicity of HIV-1 vaccines. Our pTHGag vaccine efficiently primed the immune response of the baboon to a boost with Gag VLPs, allowing the development of a strong and broadly directed cellular immune response and antibody response. These data suggest that the baboon is a NHP that can provide useful immunogenicity data prior to considering vaccines for clinical trials.

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To SIV antigens in the rhesus macaque model that is capable of limited replication-incompetent adenovirus type 5 vectors expressing a recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J Virol 77, 7663–7668.


