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Broad, high-magnitude and multifunctional CD4$^+$ and CD8$^+$ T-cell responses elicited by a DNA and modified vaccinia Ankara vaccine containing human immunodeficiency virus type 1 subtype C genes in baboons

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Candidate human immunodeficiency virus (HIV) vaccine regimens based on DNA boosted with recombinant modified vaccinia Ankara (MVA) have been in development for some time, and there is evidence for improved immunogenicity of newly developed constructs. This study describes immune responses to candidate DNA and MVA vaccines expressing multiple genes (*gag*, *RT*, *tat*, *nef* and *env*) from HIV-1 subtype C in chacma baboons (*Papio ursinus*). The vaccine regimen induced (i) strong T-cell responses, with a median of 4103 spot forming units per 10^6 peripheral blood mononuclear cells by gamma interferon (IFN-γ) ELISPOT, (ii) broad T-cell responses targeting all five vaccine-expressed genes, with a median of 12 peptides targeted per animal and without any single protein dominating the response, (iii) balanced CD4$^+$ and CD8$^+$ responses, which produced both IFN-γ and interleukin (IL)-2, including IL-2-only responses not detected by the ELISPOT assay, (iv) vaccine memory, which persisted 1 year after immunization and could be boosted further, despite strong anti-vector responses, and (v) mucosal T-cell responses in iliac and mesenteric lymph nodes in two animals tested. The majority of peptide responses mapped contained epitopes previously identified in human HIV infection, and two high-avidity HIV epitope responses were confirmed, indicating the utility of the baboon model for immunogenicity testing. Together, our data show that a combination of DNA and MVA immunization induced robust, durable, multifunctional CD4$^+$ and CD8$^+$ responses in baboons targeting multiple HIV epitopes that may home to mucosal sites. These candidate vaccines, which are immunogenic in this pre-clinical model, represent an alternative to adenoviral-based vaccines and have been approved for clinical trials.

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INTRODUCTION

Two main hypotheses are currently being tested in the human immunodeficiency virus (HIV) vaccine development field. The first, that a vaccine able to induce neutralizing antibodies will protect against infection or viral replication, has experimental support from both non-human primate and human trials (Veazey *et al.*, 2003; Emini *et al.*, 1992; Trkola *et al.*, 2005). The goal for this approach is the generation of sufficiently potent neutralizing antibodies at the site of HIV transmission (Haynes & Montefiori, 2006). The challenge is designing an immunogen capable of inducing high titre, broadly cross-neutralizing antibodies to multiple clades of HIV. The second hypothesis, that a vaccine able to generate a T-cell response will protect against disease progression by limiting viral replication, is currently being tested in clinical trials (HVTN, 2007). Original observations supporting the 'T-cell vaccine' concept were the correlation of the CD8$^+$ T-cell response with a lowering of peak viraemia in acute HIV

infection (Borrow *et al.*, 1994; Koup *et al.*, 1994), and experiments in non-human primates, where depletion of $CD8^+$ T cells resulted in uncontrolled viral replication after infection (Jin *et al.*, 1999; Schmitz *et al.*, 1999). Several features of the T-cell response to HIV have now been correlated with control of viral replication. These include preserved proliferative capacity (Day *et al.*, 2007; Migueles *et al.*, 2002; Rosenberg *et al.*, 1997) and the ability to secrete multiple cytokines (CKs) (Betts *et al.*, 2006; Harari *et al.*, 2004; Kannanganat *et al.*, 2007b). The specificity of the HIV proteins targeted by the T-cell response appears to be important, with a greater breadth of $CD8^+$ epitopes targeted in Gag (Geldmacher *et al.*, 2007; Kiepiela *et al.*, 2007; Pereyra *et al.*, 2008) or high-avidity $CD8^+$ epitopes (Almeida *et al.*, 2007) correlating with greater viral control. These and other features may be important attributes of a successful T-cell vaccine.

An adenovirus serotype 5 (Ad5)-vectored HIV vaccine recently failed to protect against infection in a Phase IIb trial (the 'STEP' trial), and an enhanced risk of infection was found in men who were uncircumcised as well as in individuals with existing anti-Ad5 vector immunity (Sekaly, 2008). The latter observation has prompted concerns regarding the potential utility and safety of the 'T-cell vaccine' concept. However, it remains to be established whether this trial represents a failure of the T-cell vaccine concept, a failure of the Ad5 vector approach or an individual product failure. Establishing any role of the vector in transmission enhancement is of critical importance as other trials with a promising and highly immunogenic DNA–Ad5 vaccine regimen (Duerr *et al.*, 2006) have currently been put on hold (Cohen & Lester, 2007). Previous large-scale trials with a canarypox vector (ALVAC) did not show any evidence of enhanced HIV transmission (Sekaly, 2008), suggesting that this may not be a feature of other viral vector platforms.

Modified vaccinia Ankara (MVA) has been in development as an alternative viral vector platform for the past decade. Initial enthusiasm for MVA was dampened by the low immunogenicity results in humans of one MVA construct (Hanke *et al.*, 2007). A subsequent smaller trial of the same construct demonstrated higher immunogenicity (Goonetilleke *et al.*, 2006). Promising newly developed MVA constructs are emerging, with high frequencies of responders (62–92 %) at higher doses (Brave *et al.*, 2007; Ramanathan *et al.*, 2007; Vasan *et al.*, 2007). Five different recombinant MVA vaccines expressing HIV genes are currently being evaluated in early phase trials in the USA, Sweden, Tanzania, Brazil and India (IAVI, 2008). The constructs are based on various HIV subtypes, express multiple genes from HIV, and are being tested in combination with other vectors. More immunogenicity data from different MVA–HIV constructs is needed to determine whether this vector platform does indeed hold promise as a vaccine for HIV. It is important to note that pre-existing anti-vector immunity, a problem for adenovirus vectors, is not an issue for MVA, since smallpox vaccination ceased in the 1970s so the majority of young people who would be the target population for an HIV vaccine do not have vaccinia antibodies (Sekaly, 2008).

The latest global figures for new acquisition of HIV show that of the estimated 2.5 million new infections worldwide, 1.7 million of these are in Africa, where AIDS remains the leading cause of mortality (UNAIDS, 2007). The major epidemics in southern Africa and India are driven by HIV-1 subtype C, which also accounts for 50 % of infections worldwide (Hemelaar *et al.*, 2006). The need for an HIV vaccine to prevent new infections in Africa remains critical.

We have described previously the construction of DNA and MVA vaccines based on HIV-1 subtype C (Burgers *et al.*, 2006, 2008). SAAVI DNA-C consists of two DNA plasmids expressing Gag, RT, Tat and Nef as a fusion protein, and a truncated Env, respectively. The sequences were derived from recently transmitted subtype C isolates, chosen for their close homology to a South African consensus sequence (Williamson *et al.*, 2003). The SAAVI MVA-C vaccine consists of a single, stable recombinant MVA expressing identical genes. Both alone and in combination, the DNA and MVA vaccines generate high-magnitude cellular immune responses in mice (Burgers *et al.*, 2006, 2008; Shephard *et al.*, 2008). These candidate vaccines are designed to generate T-cell immunity, but differ from the Ad5-based candidate vaccine tested in the STEP trial in that the regimen is a prime–boost, the vaccines include HIV Env, and the boost is vectored by an MVA recombinant. Here, we describe the immune responses generated by the candidate vaccines in non-human primates, and show that the T-cell responses generated are broad, strong, durable, multifunctional and balanced in both their specificity and phenotype.

METHODS

Animals. Nine wild-caught chacma baboons (*Papio ursinus*) housed in the South African Medical Research Council (MRC) Delft Primate Facility were used in this study. All procedures were approved by the University of Cape Town's Animal Research Ethics Committee.

Vaccines and immunizations. The DNA (termed SAAVI DNA-C) and recombinant MVA (rMVA) (termed SAAVI MVA-C) vaccine constructs used in this study have been described previously (Burgers *et al.*, 2006, 2008). Animals were divided into two groups. Six animals (515, 524, 531, 549, 575 and 630) received SAAVI DNA-C vaccine and three (533, 623 and 629) received empty vector DNA. Animals were inoculated bilaterally in the quadriceps muscle with 4 mg DNA three times, at 1 month intervals. Thirty-three weeks later, all animals were boosted with 10^9 p.f.u. rMVA, given intramuscularly as two 1 ml inoculations. This was followed by a second rMVA boost 8 weeks later. Four animals received a third dose of rMVA at week 112.

Routine monitoring of the colony for tuberculosis identified a *Mycobacterium tuberculosis* (TB) outbreak at week 63 of the study. Three animals in this study (524, 531 and 629) were found to be purified protein derivative (PPD) skin test positive and were killed. Animal 630 was also killed at this time, although there was no evidence of TB.

Peripheral blood mononuclear cells (PBMC) and lymph node processing. Blood (20–60 ml) was collected by venipuncture and PBMC were isolated by Ficoll density centrifugation. PBMC were cryopreserved in 90 % fetal calf serum, 10 % DMSO. Iliac (ILN) and mesenteric lymph nodes (MLN) were removed from two animals (629 and 630) at euthanasia, and cells were flushed out gently in RPMI 1640, then cryopreserved as before. All immunological assays were performed on cryopreserved cells that were thawed and rested overnight.

Peptides. Overlapping peptides (15–18 mers) spanning the five vaccine-expressed HIV-1 subtype C genes were used for ELISPOT and intracellular cytokine staining (ICS) assays. Peptides have been described previously (Masemola *et al.*, 2004). Ten peptide pools were made: three Gag, three Env, two Pol, and one each for Tat and Nef. Pools contained 12–50 peptides and were used at 1 μg ml^{-1}. Results are presented as cumulative responses of the individual pools (e.g. Gag responses are a sum of three Gag pools). For mapping of individual peptides, a pool-matrix design was used in the ELISPOT assay (Masemola *et al.*, 2004), and reactive peptides were then confirmed. Recognition of two adjacent peptides was considered detection as a single epitope. Optimal epitope peptides were generously provided by Nicole Frahm and Christian Brander (Partners AIDS Research Center, USA).

Gamma interferon (IFN-γ) ELISPOT assay. Secretion of IFN-γ in response to peptides was measured by the ELISPOT assay, as described previously (Masemola *et al.*, 2004). Briefly, MAIP 96-well plates (Millipore) were coated with anti-IFN-γ (5 μg ml^{-1}, 1-D1K; Mabtech) overnight, and washed and blocked the following day. Peptide pools or single peptides were added in triplicate and PBMC were plated at 100 000 cells per well. Plates were incubated for 22–24 h at 37 °C and the following day, after washing, biotin-labelled anti-IFN-γ (7-B6-1; Mabtech) was added at 2 μg ml^{-1}. Plates were incubated for 2 h at 37 °C then washed and streptavidin-horseradish peroxidase (BD Biosciences) was added. NovaRed substrate (Vector Laboratories) was used to develop spots. Plates were scanned and counted using a CTL Analyser (Cellular Technology) and Immunospot version 3.0 software. Values are reported after the subtraction of background (cells and media) and expressed as net spot-forming units (s.f.u.) per 10^6 cells. The criteria for a positive result were: three times greater than background, and $\geqslant$80 net s.f.u. per 10^6 cells. This cut-off was determined by calculating the mean +4 SD of the response of pre-immune PBMC to the peptide pools. Background was typically <20 s.f.u. per 10^6 PBMC. Because cumulative responses to peptide pools are reported, all individual pool responses falling below the cut-off were set to 0.

ICS. The production of intracellular IFN-γ and interleukin (IL)-2 in response to peptide stimuli was measured by ICS and flow cytometry. The following staining panel was used: anti-CD3-fluorescein isothiocyanate (FN18; Biosource), anti-CD8-PerCPCy5.5 (SK1), anti-IFN-γ-phycoerythrin (4SB3), anti-IL-2-allophycocyanin (MQ1-17H12; all BD Biosciences). Briefly, 1×10^6 PBMC were stimulated with either peptide pools or staphylococcal enterotoxin B (Sigma) or left unstimulated. All tubes received CD49d and CD28 (2 μg ml^{-1}; BD Biosciences). Tubes were incubated at 37 °C, brefeldin A (10 μg ml^{-1}; Sigma) was added after 1 h, and cells were incubated for a further 5 h. Cells were washed, stained with anti-CD8, washed and resuspended in FACS Lyse (BD Biosciences) and then Perm Solution 2 (BD Biosciences). Cells were then stained with anti-CD3, anti-IL-2 and anti-IFN-γ. Cells were resuspended in Cellfix (BD Biosciences), 100 000–200 000 lymphocyte-gated events were acquired on a FACSCalibur (BD Biosciences), and data were analysed using FlowJo version 5.7.1 (Treestar). The gating strategy was as follows: lymphocytes, CD3$^+$, CD8$^+$ and CD8$^-$. CD8$^-$ cells were classified as CD4$^+$ cells. Values are reported as net percentage of total CK-secreting CD3$^+$CD8$^+$ or CD3$^+$CD8$^-$ cells. A positive response had to be at least twice that for the background (unstimulated) tube, as well as $\geqslant$0.05 %. Typical background staining values for CD8$^+$ and CD8$^-$ cells were <0.05 % for IFN-γ, 0.2 % for IL-2 and 0 for dual expression of IFN-γ and IL-2. For determining the phenotype of cells and investigating lymph node immunity, anti-CD4 replaced anti-IL2.

Anti-gp120 ELISA. HIV-1 gp120 antibodies were detected by using a standard ELISA as described previously (Burgers *et al.*, 2006; Shephard *et al.*, 2008). HIV-1 subtype C gp120 (Lian *et al.*, 2005) was kindly provided by Susan Barnett (Chiron Corporation). End-point titres were defined as the reciprocal of the highest dilution whose OD value was threefold over that of the background pre-immunization sera at the lowest dilution.

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 4. Median values, interquartile ranges and ranges are presented. Correlations were performed with Spearman Rank, and the Mann–Whitney test was used for differences between groups. A P value <0.05 was considered significant.

RESULTS

Cellular immune responses to DNA–MVA vaccination are broad and strong

The immunogenicity of vaccine constructs SAAVI DNA-C and MVA-C was tested in baboons using the IFN-γ ELISPOT assay. Six animals received three doses of DNA, followed by two doses of MVA (Fig. 1). A further three animals received empty vector DNA and two doses of MVA ('MVA-only' group). Cellular immune responses were monitored longitudinally using peptide pools covering Gag, Pol, Env, Nef and Tat. As shown in Fig. 2(a), the DNA vaccine construct alone was poorly immunogenic. After boosting with MVA, however, five of six vaccine recipients generated high-magnitude cellular responses. The responses in the MVA-only group differed markedly in magnitude, breadth and kinetics compared with those of the DNA–MVA group. A significant difference was observed between the median magnitude of responses in DNA–MVA responders and MVA-only animals (4103 versus 272 net s.f.u. per 10^6 PBMC, P=0.03). DNA–MVA immunization also induced broad T-cell responses (Fig. 2b). The breadth of responses differed significantly between the two groups (responses to seven of 10 peptide pools versus single-pool responses, P=0.03). Responses

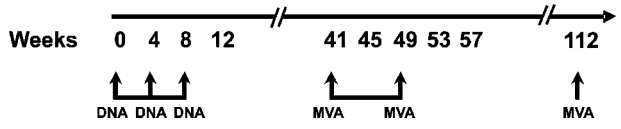

Fig. 1. Immunization schedule for baboons. Six animals received three priming immunizations of SAAVI DNA-C (4 mg) 4 weeks apart, while three animals received empty vector DNA. At week 41 and 49, all animals received recombinant SAAVI MVA-C (10^9 p.f.u.). Sixty-three weeks later, four animals received a third boost with recombinant MVA (week 112).

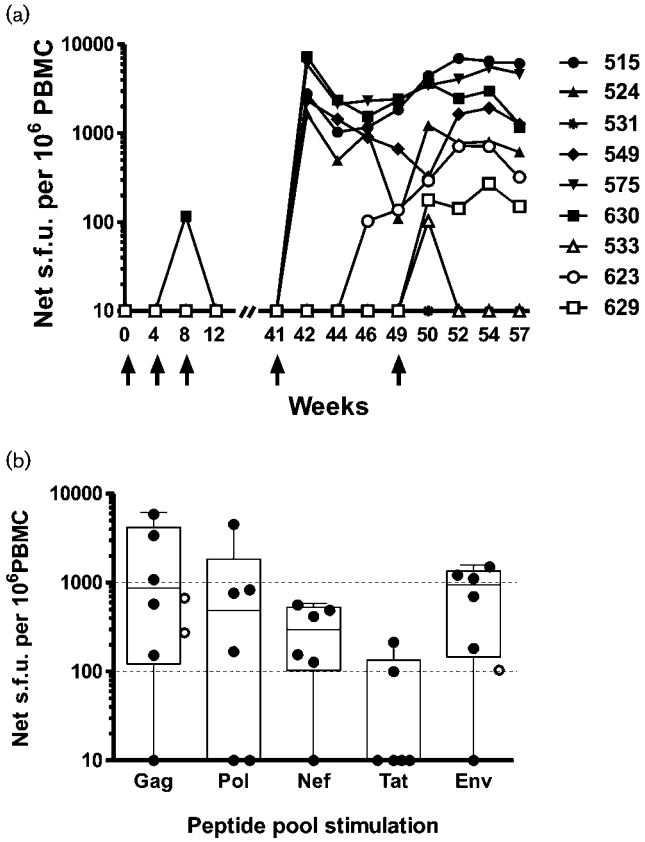

Fig. 2. IFN-γ ELISPOT responses to vaccine antigens. (a) Kinetics of the total magnitude of IFN-γ ELISPOT responses over the vaccination period for individual study animals. Arrows indicate where immunizations were given. (b) Cumulative IFN-γ ELISPOT responses to peptide pools spanning Gag, Pol, Nef, Tat and Env are shown. Medians, interquartile ranges and ranges are represented for DNA–MVA-vaccinated animals. Results are reported as net s.f.u. per 10^6 PBMC at peak time points. Filled symbols represent DNA–MVA-vaccinated animals and open symbols indicate animals that received only recombinant MVA.

were detected to all proteins expressed from the vaccines, with Gag and Pol having the highest magnitude responses in individual DNA–MVA animals (up to 5892 net s.f.u. per 10^6 PBMC), and Env the highest median responses (913 net s.f.u. per 10^6 PBMC). Of the five DNA–MVA vaccine responders, all animals recognized four or all five HIV proteins in the vaccines, whilst MVA-only animals had single protein responses, to either Gag or Env (Fig. 2b). The striking boosting effect of MVA shows clearly that the DNA prime did indeed induce an immune response, even though it was below the level of detection in our assays. Thus, the combination of DNA and MVA vaccine constructs induced broad, high-magnitude IFN-γ T-cell responses.

The kinetics of the immune response were remarkably uniform. In four of five DNA–MVA responders, the response peaked 1 week after the first MVA immunization, after which it reduced dramatically, by a mean of 64 % 2 weeks later (Fig. 2a). The boosting and contraction effect was delayed and reduced after the second MVA immunization, and the magnitude did not exceed the peak response after the first MVA in any of these four animals. Thus, although a second dose of MVA did not further boost responses in the majority of DNA–MVA responders, it may serve to induce a population of longer-lived memory cells.

Induction of polyfunctional CD4$^+$ and CD8$^+$ T-cell responses

We next sought to determine whether the vaccine-induced responses detected in the ELISPOT assay were mediated by CD8$^+$ or CD8$^-$ (CD4$^+$) T cells, by ICS for IFN-γ and IL-2. Representative flow cytometry plots from peak response time points are presented in Fig. 3(a), demonstrating three functional T-cell populations, namely those cells producing IFN-γ alone, IL-2 alone and both CKs simultaneously. DNA–MVA vaccines elicited HIV-specific CD4$^+$ and CD8$^+$ T-cell responses capable of producing IFN-γ, IL-2 and both CKs (Fig. 3b).

Only CD8$^+$ T-cell responses were detected in two of the three MVA-only animals. There was a highly statistically significant positive correlation between the CD3$^+$ IFN-γ responses detected by ICS and those detected by the IFN-γ ELISPOT assay (r=0.86, P<0.0001, data not shown). The CD8$^+$ compartment was responsible for 80 % of the total IFN-γ response in the DNA–MVA group. The CD4$^+$ compartment, in turn, was responsible for most (60 %) of the IL-2 response. Thus, HIV-specific CD8$^+$ cells produced threefold more IFN-γ than CD4$^+$ T cells, and CD4$^+$ cells produced 2.7-fold more IL-2 than IFN-γ. Dual-CK-producing cells accounted for roughly half of the IFN-γ-producing CD4$^+$ population and a third of the IL-2-producing cells. In contrast, the CD8$^+$ T-cell population consisted of far fewer dual-CK-producing cells. This indicates a greater heterogeneity in the CD4$^+$ compartment, with CD4$^+$ T cells producing a more polyfunctional response. Whilst there was a trend towards a higher magnitude of CK$^+$ CD4$^+$ T cells specific for Gag and Env compared with CD8$^+$ T cells, these differences were not significant (Fig. 3c). The median of the total CK$^+$ population was similar in magnitude for CD8$^+$ and CD4$^+$ T cells in the DNA–MVA-vaccinated group (Fig. 3c, inset). When calculating the average proportion of the response to the vaccine-expressed genes, it was evident that response was not dominated by any single vaccine antigen (Fig. 3d).

Interestingly, 11 positive peptide pool responses in the CD4$^+$ compartment, directed mostly at the Env protein, produced IL-2 only, in the absence of any IFN-γ (data no shown). These responses would not have been detected using the IFN-γ ELISPOT assay. This emphasizes the limitation of screening for vaccine responses using the IFN-γ ELISPOT assay alone and the need to measure CKs in addition to IFN-γ when evaluating candidate vaccines,

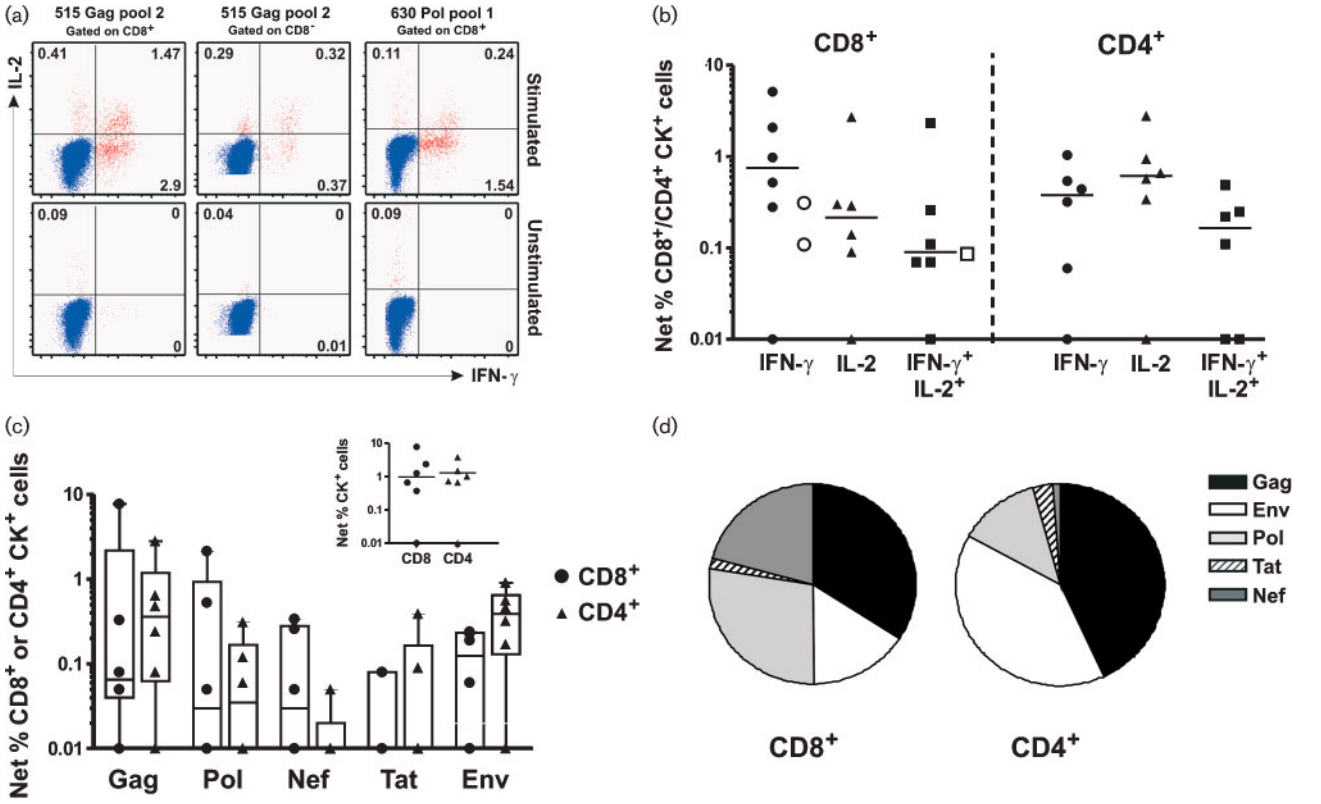

Fig. 3. HIV-specific IFN-γ and IL-2 production by T cells induced by vaccination. ICS and flow cytometry were used to identify vaccine-induced HIV-specific immune responses using peptide pools spanning Gag, Pol, Nef, Tat and Env. Cells were gated on lymphocytes, $CD3^+$ and $CD8^+$ and $CD8^-$. $CD8^-$ cells were classified as $CD4^+$ cells. (a) Representative flow cytometry plots. (b) IFN-γ^+, IL-2^+ and IFN-γ^+IL-2^+ dual-producing $CD8^+$ and $CD4^+$ T cells. Filled symbols represent DNA–MVA-vaccinated animals and open symbols indicate animals that received only recombinant MVA. (c) CK^+ (IFN-γ or IL-2) cells specific for the various vaccine antigens for the DNA–MVA-vaccinated group. Inset: total magnitude of CK^+CD8^+ or $CD4^+$ T cells per animal. (d) Mean proportion of response directed to each of the vaccine antigens in CK^+CD8^+ or $CD4^+$ T cells. All values are shown as net percentage of total CK-secreting $CD3^+CD8^+$ or $CD3^+CD8^-$ cells. ICS responses are reported at time points corresponding to peak responses obtained in the ELISPOT assay prior to the third MVA immunization. Lines indicate medians, and (c) includes interquartile ranges and ranges.

particularly for detecting $CD4^+$ responses. These data demonstrate that the DNA–MVA vaccine regimen is capable of inducing a balanced $CD8^+$ and $CD4^+$ T-cell response, and that HIV-specific cells produce IFN-γ and IL-2.

Mapping of peptide responses

In order to characterize the nature of the cellular responses generated by the vaccines further, the IFN-γ ELISPOT assay and a matrix of peptide pools was used to map the peptide specificities of reactive T-cell populations. At least 34 different HIV peptides were recognized by vaccinated animals (Table 1). DNA–MVA-vaccinated animals responded to a median of 12 (range 7–13) peptides. There were 11 peptide responses identified for Env and Pol each, nine for Gag, two for Nef and one for Tat. Animals typically had a mixture of a single high-magnitude-dominant response for Gag, Pol or Env, and numerous subdominant responses of lower magnitude. There was no evidence of preferential targeting of proteins. This heterogeneity of response is likely to be due to the outbred nature of the animals used in the study. Despite this, two immunodominant responses in Gag and Env were recognized in four of five baboons, and a further 13 peptides (40 % of identified peptides) were recognized by two or more animals. It is important to note that these responses do not represent the full breadth of vaccine-induced responses, as the responding peptides responsible for IL-2-only responses were not mapped by the IFN-γ ELISPOT. Where cells were available, the phenotype of the response was determined using ICS. Ten peptide responses were identified as being mediated by $CD8^+$ T cells, and five as being mediated by $CD4^+$ T cells.

Two-thirds of the peptides (23 of 34) contained epitopes that have been identified in human HIV infection. In

Table 1. Confirmed peptide responses in vaccinated animals

Protein	Confirmed peptide sequence*†	Animal no.	Net s.f.u. per 10^6 PBMC‡	Phenotype§	Selection of CD8 or CD4 epitopes within sequence with restricting HLA (if known)‖
Gag					
p17	^{37}ASRELERFALNPGLL^{51}	515, 549	110	**CD8**	No previously described CD8 epitopes
p17	^{63}QLQPALQTGTEELRSLY^{79}	575	146	ND	G**S**EELRSLY(A1)
p24	^{149}PRTLNAWVKVIEEKAF^{164}	575, 630, 515	293	ND	RTLNAWVKV(A2); VKVIEEKAF(B*1503)
p24	^{163}AFSPEVIPMFTALSEGA^{179}	549	270	ND	FSPEVIPMF(B57); EVIPMF**S**AL(A*2601)
p24	^{257}PVGDIYKRWIILGLNKIV^{274}	575, 549	248	ND	GDIYKRWII(B*0801); IYKRWIILGL(A*2402); KRWIILGLNK(B*2705)
p24	^{265}WIILGLNK**IVRMYSPVSI**^{282}	549	300	**CD4**	NKIVRMYSP**T**SI(DRB1*1101, DRB1*1501, DRB5*0101)
p24	^{296}**YVDRFFKTLR**AEQAT^{310}	623, 629, 515, 549	495	**CD8**	YVDRFFKTL(A26, B70, B*1503); DRFFKTLRA(B*1403)
p24	^{332}TILRALGPGATLEEM^{346}	575	230	ND	**K**ALGP**A**ATL
p24	^{368}**SQTNSGNIMMQ**RSNF^{383}	575	5900	**CD8**	No previously described CD8 epitopes
Nef					
	^{50}**HNNPDCAWLQA**QEEE^{64}	575	120	ND	**AT**N**A**DCAWL(A2)
	^{181}LKWVFDSSLARRHLA^{194}	524, 549	605	**CD8**	L**E**W**R**FDS**R**L(A2); **E**W**R**FDS**R**L(B8); W**R**FDS**R**LA**F**(B*1503)
Pol					
Pr	^{124}GKKAIGTV**LVGPTPVNII**^{141}	575, 630	210	ND	LVGPTPVNI(A*0201)
Pr	^{147}TQLGCTLNFPISPIETV^{163}	630	170	ND	CTLNFPISPI(A2)
RT	^{183}EVKIKA**LTAICEEMEK**^{198}	630	120	ND	AL**VE**IC**T**EM(A*0201); AL**VE**IC**T**EMEK(A*0301)
RT	^{202}ITKIGPENPYNTPIFAIK^{219}	515, 524	80	**CD4**	No previously described CD4 epitopes
RT	^{218}IKKEDSTK**WRKLVDFREL**^{235}	630	220	ND	No previously described epitopes
RT	^{272}SVPLDEGFRKYTAFTI^{287}	630, 524	1705	**CD8**	SVPLDE**S**FRK(A11); VPLDE**D**FRKY(B*3501)
RT	^{295}PGIRYQYNVLPQGWK^{309}	524	80	**CD8**	No previously described CD8 epitopes
RT	^{395}TVQPIQLP**EKDSWTVNDI**^{412}	630, 515	158	**CD8**	I**V**LPEKDSW(B*5701)
RT	^{492}WTYQIYQEPFKNLKTGKY^{509}	515	95	**CD8**	IYQEPFKNL(A*2301); IYQEPFKNLK(A*1101)
RT	^{544}**FRLPIQKETW**EIWWTDYW^{561}	630, 524	570	**CD8**	PIQKETWE**T**W(A*3201)
RT	^{552}TWEIWWTDYWQATWIPEW^{569}	524	80		TWE**T**WWT**E**YW(B44)
Env					
gp120	^{33}NLWVTIYYGVPVWREAK^{49}	575, 630, 549	186	ND	LWVT**V**YYGV(A*0201); VT**V**YYGVPVW**K**(A*1101); T**V**YYGVPVW**K**(A*0301)
gp120	^{49}REAKTTLFCASDAKAYDR^{63}	630	130	**CD4**	No previously described CD4 epitopes
gp120	^{88}NVTENFNMWKNDMVDQMH^{105}	575, 630, 549	146	ND	VTENFNMWKN(A11, A68)
gp120	^{116}LKPCVKLTPLCVTLK^{130}	575, 524, 515, 549	286	ND	KPCVKLTPLC(B7); KLTPLCVTL(A*0201)
gp120	^{143}YNGSDTNDMRNCSFNTTTEI^{165}	575	439	ND	NCSFN**IS**T**S**I(Cw*08)
gp120	^{172}VYALFYKPDIVPINESEY^{189}	630	210	ND	No previously described epitopes
gp120	^{207}KVSFDPIPIHYCAPAGYA^{224}	630	410	**CD4**	No previously described CD4 epitopes
gp120	^{215}IHYCAPAGYAILKCNNK^{231}	515	80	ND	No previously described epitopes
gp120	^{474}NMKDNWRSELYKYKVVEI^{491}	549	650	ND	No previously described epitopes
gp41	^{561}AQQHMLQLTVWGIKQL^{576}	549	150	**CD8**	**L**LQLTVWGI(A2)
gp41	^{587}**LKDQQLLGLW**GCSGKLI^{603}	575, 549	180	**CD4**	No previously described CD4 epitopes
Tat					
	^{24}NCYCKHCSYHCLVCFQTK^{41}	575, 515	333	ND	NCYCK**K**C**C**Y(A*2902)

*Amino acids according to location in HXB2.

†Amino acids in bold indicate overlap with adjacent positive peptide.

‡Median values are presented where more than one animal had a response.

§As determined by ICS; ND, not determined.

‖Amino acids in bold indicate mismatches with the subtype C peptide.

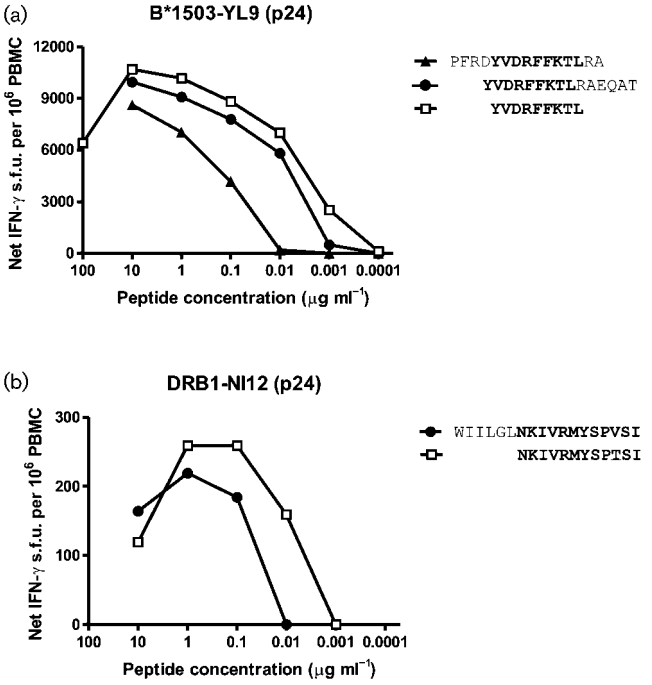

Fig. 4. Functional avidity of reactive epitopes in vaccinated animals. Two optimal epitopes identified in human HIV infection were confirmed in vaccinated baboons. Ten-fold serial dilutions of peptides were tested, ranging from 100 or 10 μg ml^{-1} to 10 pg ml^{-1}. Functional avidity was defined as the concentration of peptide that gave half the maximum response in an IFN-γ ELISPOT assay, expressed as net s.f.u. per 10^6 PBMC. (a) Responses to two overlapping 15 mer peptides in p24 and the optimal YL9 peptide restricted by class I HLA B*1503. (b) Responses to an 18 mer peptide in p24 and the optimal class II HLA DRB1 epitope NI12.

humans, the majority of these epitopes are restricted by HLA-A and -B molecules, suggesting that baboons present HLA-A- and HLA-B-like epitopes. Two of the 23 epitopes previously identified in human HIV infection were confirmed using optimal peptides (Fig. 4a and b). Both epitopes are found in Gag p24, and restricted by HLA B*1503 or DRB1. Responses to the optimal epitopes were greater than responses to the longer overlapping peptides, and optimal epitopes displayed high functional avidity, with half-maximal responses at 1–10 pg peptide ml^{-1}.

Long-lived memory responses to vaccination

The longevity of the immune response generated by a vaccine may be critical for its success, given that exposure to HIV may occur years after vaccination. In four animals, HIV-specific T cells persisted 1 year after the second MVA immunization, in some cases at high levels (Fig. 5). To investigate the effect of further boosting of the immune response, a third MVA immunization was given. In all four animals, boosting induced responses that were higher than the previous peak responses. Mapping of individual peptide responses in DNA–MVA vaccinees revealed that this was not due to a broadening of the response, but in most cases to a considerable increase in the magnitude of a single response that had dominated previously. For example, a single p24-peptide-specific response in animal 575 was responsible for approximately 90 %, or 7993 net s.f.u. per 10^6 PBMC, of the response. Interestingly, in one animal (549) an initially subdominant Nef response dominated after the third MVA dose, supporting a role for epitope competition for shared major histocompatibility complex (MHC) molecules. In contrast, in the single MVA-only animal tested (623), a third dose of MVA not only increased the magnitude of the response sixfold with respect to the previous peak response, but also considerably

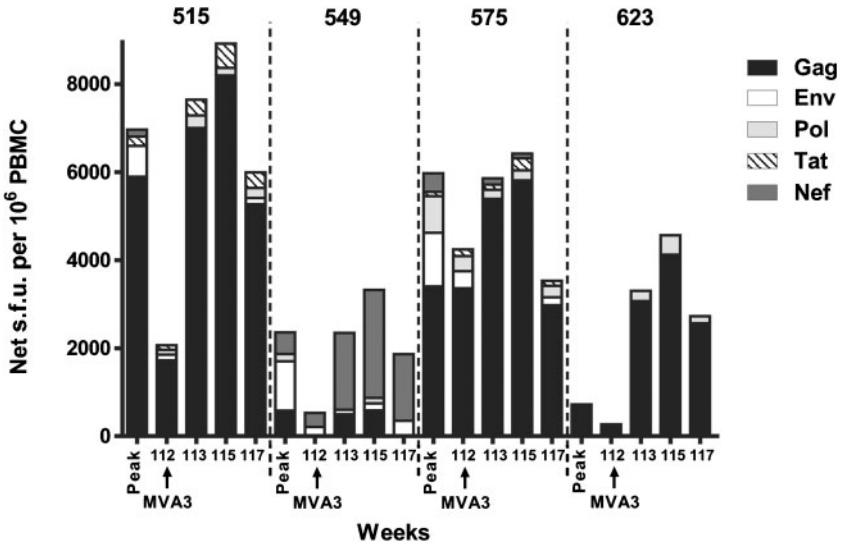

Fig. 5. Vaccine memory persists and can be boosted. Four animals (515, 549, 575 and 623) received a third dose of MVA (10^9 p.f.u.) at week 112, 1 year after the second MVA immunization. Cumulative IFN-γ ELISPOT responses to peptide pools spanning Gag, Pol, Nef, Tat and Env are shown, reported as net s.f.u. per 10^6 PBMC. Peak responses prior to the third immunization are shown, as well as responses on the day of immunization and the peak response thereafter, which occurred 3 weeks post-vaccination in all animals.

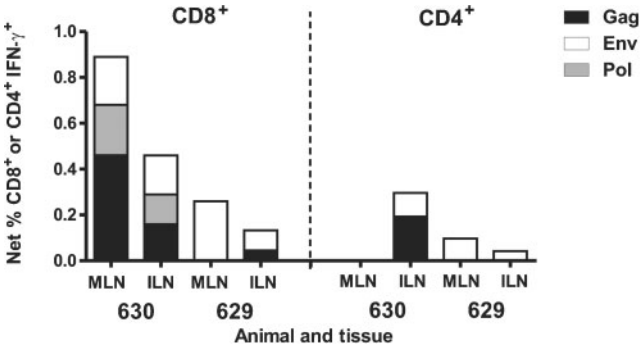

Fig. 6. Mucosal T-cell responses to vaccine antigens present in MLN and ILN in vaccinated animals at week 65. Responses were measured to peptide pools using ICS as described in Methods, and cumulative responses to HIV proteins are shown. The net percentage of $CD8^+IFN\text{-}\gamma^+$ and $CD4^+IFN\text{-}\gamma^+$ cells is shown for animal 630, which received DNA and MVA, and 629, which received MVA only.

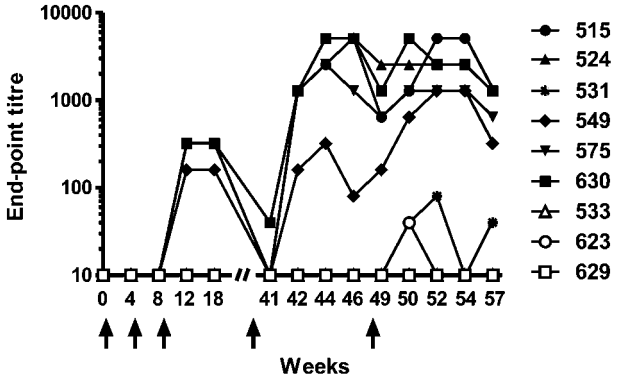

Fig. 7. Antibody responses to HIV gp120. Sera from animals immunized with SAAVI DNA-C and MVA-C (filled symbols) or MVA-C alone (open symbols) were tested for antibody responses to HIV-1 subtype C gp120. Immunizations are indicated by arrows. Antibody responses were evaluated by ELISA and values represent end-point serum titres.

broadened the response, with an additional Gag and two Pol peptide pool responses being generated.

HIV-specific immunity in mucosal lymph nodes

The presence of vaccine-induced immunity in mucosal draining lymph nodes was investigated in two animals, one of which received MVA alone (629) and the other DNA–MVA (630). Mucosal immunity was demonstrated by the identification of HIV-specific responses in the ILN and MLN of both animals (Fig. 6). In animal 630, the specificity of the response in ILN lymphocytes reflected responses observed in PBMC. $CD8^+$ responses were present in MLN, whilst both $CD8^+$ and $CD4^+$ responses were detectable in ILN. Interestingly, an Env response that had not been identified previously in PBMC was present in lymph nodes of animal 629. Whilst this observation was limited to two animals, the data highlight the potential of systemic immunization with these vectors to induce HIV-specific immune cells that may readily traffic to the gut and genital tract.

Antibody responses to Env but no neutralization

In order to investigate whether the vaccine constructs elicited an antibody response to HIV Env, sera from vaccinated baboons were tested for the presence of antibodies to HIV-1 subtype C gp120 by ELISA. Binding antibodies to gp120 were detectable in all animals that received both vaccines (Fig. 7). Weak titres present in five of six animals after DNA immunization were boosted 12-fold after MVA immunization, and peak titres ranged from 80 to 5160. A weak and transient antibody response was detectable in one of three animals that received SAAVI MVA-C in the absence of DNA priming. We next investigated whether the sera had the ability to neutralize autologous and heterologous viruses, using a pseudovirus neutralization assay (Li *et al.*, 2005). No neutralizing activity was detected in any of the vaccinated animals (data not shown). Thus, moderate titres of Env-binding antibodies lacking neutralizing activity were generated in baboons given the combination of SAAVI DNA-C and MVA-C candidate vaccines.

DISCUSSION

We report here on the immunogenicity of DNA and MVA vaccines containing multiple genes from HIV-1 subtype C in a small group of outbred baboons. The DNA–MVA vaccination regimen induced strong IFN-γ and IL-2 responses with a balanced distribution in the $CD8^+$ and $CD4^+$ T-cell compartments. Responses were broad and there was evidence that the vaccine regimen could induce mucosal immunity as well as long-lasting responses, present over a year after vaccination.

Although rhesus macaques are the animal of choice for immunogenicity testing of HIV vaccines, it is unlikely that immune responses in baboons differ markedly from those of macaques. Comparison of responses to an identical Ad5-Gag vaccine in macaques and baboons showed no significant differences (Casimiro *et al.*, 2003a, b). Like macaques, baboons possess MHC class I A and B alleles, but lack HLA-C allele homologues (Sidebottom *et al.*, 2001). Phylogenetic analysis reveals that *Papio* A and B alleles cluster closely with macaque alleles (Prilliman *et al.*, 1996). Baboons also have homologues of human class II DRB and DQ (Gaur *et al.*, 1997, 1998). Indeed, we demonstrate here that baboons present HIV peptides containing epitopes detected in human HIV infection, as has been observed in macaque vaccine studies (Amara *et al.*, 2005). Seven of these were found to be frequently

targeted immunodominant peptides across multiple ethnicities (Frahm *et al.*, 2004), and we confirmed two epitopes as being identical to those recognized in humans. We also observed that baboons may present both HLA-A- and HLA-B-like epitopes, unlike macaques which were recently shown to present predominantly HLA-B-like epitopes (Hickman-Miller *et al.*, 2005). A further advantage of using baboons is their larger size, allowing greater blood sample volumes for in depth immunological evaluations. This indicates the utility of the baboon model for immunogenicity testing, and allows us to reasonably compare the responses generated in our study with vaccine studies conducted in macaques. However, the question of whether non-human primates predict immune responses in humans remains unanswered, and only when more published studies of human trials with immunogenic vaccines become available can this issue be addressed satisfactorily.

The use of a pool and matrix ELISPOT design enabled the identification of individual peptides responsible for the HIV immune responses generated. In human studies of natural HIV infection, individuals amount a median of 14 epitope responses to a consensus HIV proteome, and a median magnitude of 4245 net IFN-γ s.f.u. per 10^6 PBMC has been reported from ELISPOT assays in large cohorts (Addo *et al.*, 2003). These figures are remarkably similar to the results from our small group of vaccine responders vaccinated with 60 % of the HIV proteome and measured with autologous peptides, who recognized a median of 12 epitopes and had a median response of 4103 net s.f.u. per 10^6 PBMC. In HIV infection Gag and Nef dominate the T-cell response (Addo *et al.*, 2003; Masemola *et al.*, 2004), whilst Env, Pol and Gag had the highest breadth and magnitude of responses for our vaccine regimen. Differences in immunodominance are probably a reflection of the MHC repertoire in this group of baboons, as well as the antigen load and timing of expression from the vaccine constructs being very different from natural infection.

A number of studies have demonstrated that the production of IFN-γ does not correlate with viral control in chronic HIV infection (Addo *et al.*, 2003; Masemola *et al.*, 2004). We demonstrated the presence of $CD8^+$ and $CD4^+$ vaccine-induced T cells capable of producing IFN-γ, IL-2 and both IFN-γ and IL-2 simultaneously. In HIV infection, $CD4^+$ T cells capable of producing both IFN-γ and IL-2, as well as polyfunctional $CD8^+$ T cells, are found in greater abundance in HIV non-progressors (Betts *et al.*, 2006; Harari *et al.*, 2004). Elite controllers also possess greater numbers of $CD4^+$ and $CD8^+$ T cells secreting both IFN-γ and IL-2 (Pereyra *et al.*, 2008). These studies suggest that T cells producing multiple CKs are superior effectors, and there are now several reports showing that polyfunctional T cells produce more CK per cell, and $CD4^+$ polyfunctional cells have an increased ability to activate $CD8^+$ cells and to degranulate (Darrah *et al.*, 2007; Kannanganat *et al.*, 2007a). The production of IL-2 may be particularly important for the expansion and maintenance of the T-cell response.

Our study shows that the DNA and MVA vaccination regimen induced high-magnitude responses targeting all of the HIV antigens included in the vaccines in the baboon model. It is not known what the critical threshold of HIV-specific T cells is that will be able to protect against infection or disease, or whether the overall breadth of the immune response or the specific region of HIV that is targeted is more critical for viral control. Studies in chronic HIV infection have not been able to establish a relationship between overall breadth or magnitude of the IFN-γ T-cell response to HIV and viral control (Addo *et al.*, 2003; Masemola *et al.*, 2004). With regard to the specificity of the response, only preferential targeting of Gag, and the breadth of the Gag response, has been correlated with superior viral control in large chronic HIV cohorts, as well as in elite controllers (Kiepiela *et al.*, 2007; Geldmacher *et al.*, 2007; Pereyra *et al.*, 2008). How readily these lessons from chronic HIV infection should be applied to vaccine design and evaluation is not known. Macaques given a DNA–poxvirus vaccination regimen, which included six simian immunodeficiency virus (SIV) antigens, had reduced viraemia in the acute and chronic phases of infection and better survival in response to SIV challenge than those treated with a three-antigen vaccine (Hel *et al.*, 2006). In addition, the magnitude of pre-challenge immune responses in vaccinated animals correlated with survival advantage (Hel *et al.*, 2002). Thus, there is some evidence that breadth and magnitude may be important for vaccines.

There are multiple Ad5- and poxvirus-vector-based candidate vaccines in early or later stages of clinical trial development and there is an urgent need for direct comparative studies to be performed to rationalize further development and testing. Whilst it is not possible to directly compare vaccine approaches without performing a head-to-head study, analysis of published data indicates that the response magnitudes to the DNA–MVA vaccines presented here are in the same range as responses to candidate multigene vaccines based on DNA–MVA and DNA–Ad5 tested in macaques (Robinson *et al.*, 2007; Santra *et al.*, 2005).

Long-lived memory responses will be critical for vaccine success. The kinetics of responses in our study were similar to those in human volunteers in clinical trials with DNA and MVA vaccines (Goonetilleke *et al.*, 2006), with a peak 1 week after MVA boosting, followed by a rapid decay of >50 % by 2 weeks later. We did not measure responses 2 weeks after vaccination, and it is possible that we may have missed an even greater peak response. A second dose of MVA boosted declining responses, albeit to levels lower than peak responses after the first MVA inoculation. However, there appeared to be a delay in the decay of the responses after the second MVA, suggesting that the second inoculation may induce a population of longer-lived HIV-specific T cells, important for vaccine memory. Indeed, we were able to detect persisting responses a year after the last MVA vaccination.

In addition to T-cell responses, we also detected antibody responses to Env, although these did not possess any neutralizing ability. Whilst non-neutralizing antibodies may have some importance in controlling HIV (Montefiori *et al.*, 2007), the value of including Env may be due to the greater breadth of particularly $CD4^+$ helper responses it elicits. Several pre-clinical studies have demonstrated an advantage for disease outcome in macaques after inclusion of Env in candidate vaccines (Letvin *et al.*, 2004; Amara *et al.*, 2002). Whilst the $CD4^+$ T-cell responses mediated by Env may be advantageous for providing help for memory $CD8^+$ development, a possible drawback of the inclusion of Env in multigene vaccines is its tendency to dominate the T-cell response, as evidenced by recent DNA–NYVAC trials in both macaques and humans (Harari *et al.*, 2008; Mooij *et al.*, 2008). This was also observed for a multigene DNA vaccine (Graham *et al.*, 2006). Although the delivery vector is likely to have a greater influence on the balance of $CD8^+$ and $CD4^+$ responses than specific antigens (Mooij *et al.*, 2008), the tendency of Env responses elicited by poxvirus vectors to be skewed towards $CD4^+$ may result in reduced induction of $CD8^+$ CTLs, or the presence of $CD8^+$ T cells targeting Env rather than more conserved regions of HIV such as Gag, which may ultimately limit vaccine efficacy. An important observation was that our vaccine regimen induced responses that were not dominated by any single vaccine antigen. This may be related to expression level, since we placed *env* under the control of a weaker promoter in the recombinant MVA than the Gag, RT, Tat and Nef fusions, for reasons of stability (Burgers *et al.*, 2008).

The ability of an HIV vaccine to induce immunity in mucosal tissues such as the genital tract and gut, where transmission and initial massive replication occur, may be a key attribute for efficacy (Brenchley & Douek, 2008). We had ILN and MLN tissue available from two vaccinated animals, and the anti-HIV immunity detected here shows that vaccine memory may persist in lymph nodes, which in turn may home to mucosal tissues upon exposure to HIV. Systemic vaccination with DNA and viral vector regimens has previously been demonstrated to induce mucosal immune responses in macaques (Baig *et al.*, 2002; Stevceva *et al.*, 2002; Mattapallil *et al.*, 2006b). Reduced destruction of CD4 memory cells in mucosal tissues resulted in lower acute-phase viraemia and better survival in DNA–Ad5-vaccinated and SIV-challenged animals (Mattapallil *et al.*, 2006a, b). Development of methodology and monitoring of mucosal immunity in pre-clinical and clinical trials of candidate vaccines is imperative, and we are planning to extend these pre-clinical studies to characterize the extent of vaccine-induced immunity and persistence of HIV-specific cells in mucosal lymphoid organs and tissues.

In our vaccine regimen, DNA priming was essential for inducing strong responses post-MVA. Our studies focused on immunogenicity, and we have not performed SIV challenge. Whilst clinical trials in human volunteers need to be carried out to determine the efficacy of any candidate vaccine, important insights can be gained from studies in vaccinated macaques challenged with pathogenic SIV. Vaccine gains have been modest, with lower and/or delayed acute viraemia and better survival times after challenge in vaccinated animals compared with unvaccinated controls. These advantages have been demonstrated in an SIV-challenge model in Mamu-A*01(−) animals for, among others, DNA (Rosati *et al.*, 2005), DNA–MVA (Horton *et al.*, 2002), DNA–NYVAC (Hel *et al.*, 2006) and DNA–Ad5 vaccine regimens (Letvin *et al.*, 2006; Sun *et al.*, 2006; Mattapallil *et al.*, 2006a). Thus, poxviruses have a good record of modest effects in SIV-challenge models.

In summary, we have demonstrated good immunogenicity data for a multigene HIV-1 subtype C DNA and MVA vaccination regimen. The similarity in the magnitude, specificity and kinetics of the immune response generated in baboons to that demonstrated in previous studies in macaques and humans suggests that baboons are an acceptable immunogenicity model, and that these vaccine candidates generate similar T-cell immune responses in non-human primates to leading vaccine candidates. The MVA vaccine described in this study, and a second generation DNA vaccine consisting of an altered vector backbone (Barouch *et al.*, 2005), have been approved by the FDA for testing in a prime–boost vaccination regimen in humans. The proposed vaccination regimen for clinical trials mirrors closely that described here, where doses of 10^9 p.f.u. MVA will be given, albeit in a shorter timeframe, with a 2 month interval between primes and boosts. These candidate vaccines are scheduled to enter clinical trials in 2008 (HVTN, 2007).

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