

# Vaccine protection from CD4<sup>+</sup> T-cell loss caused by simian immunodeficiency virus (SIV) mac251 is afforded by sequential immunization with three unrelated vaccine vectors encoding multiple SIV antigens

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Candidate human immunodeficiency virus (HIV) vaccine strategies that induce strong cellular immune responses protect rhesus macaques that are infected with recombinant simian/human immunodeficiency virus SHIV89.6p from acute CD4<sup>+</sup> T-cell loss and delay progression to AIDS. However, similar strategies have not proven as efficacious in the simian immunodeficiency virus (SIV)mac model of AIDS, an infection that causes a slow, steady loss of CD4<sup>+</sup> T-cell function and numbers in rhesus macaques similar to that caused by HIV-1, the principal cause of AIDS in humans. Efforts to increase vaccine efficacy by repeated boosting with the same vector are quickly limited by rising anti-vector immune responses. Here, the sequential use of three different vectors (DNA, Semliki Forest virus and modified vaccinia virus Ankara) encoding the same SIVmac structural and regulatory antigens was investigated and demonstrated to prevent or slow the loss of CD4<sup>+</sup> T-cells after mucosal challenge with the highly pathogenic SIVmac251 strain. Of particular interest was an inverse association between the extent of T-helper 2 cytokine responses and steady-state virus load. Although limited in the number of animals, this study provides important proof of the efficacy of the triple-vector vaccine strategy against chronic, progressive CD4<sup>+</sup> T-cell loss in the rigorous SIVmac/rhesus macaque model of AIDS.

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## INTRODUCTION

To combat the rapidly spreading AIDS epidemic, a safe and effective prophylactic human immunodeficiency virus (HIV) vaccine is urgently needed (UNAIDS, 2003). Despite many efforts, an ideal vaccine candidate has not yet emerged. Infection of rhesus macaques with simian immunodeficiency virus (SIV) or, more recently, recombinant HIV/SIV (SHIV) strains has been used as a model for AIDS and has provided important information for vaccine development. A number of evolving HIV vaccine candidates are based on a two-vector prime/boost approach. Although unable to protect from infection, these have been demonstrated to protect from acute CD4<sup>+</sup> T-cell loss in the SHIV89.6p model (Robinson *et al.*, 1999; Amara

*et al.*, 2001; Barouch *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002). Prophylactic vaccine efficacy in the SHIV pre-clinical model has been correlated with either neutralizing antibodies or strong CD8<sup>+</sup> T-cell responses and, more recently, results have suggested the growing importance of T-helper (Th) cell responses in protection in this model (Jin *et al.*, 1999; Schmitz *et al.*, 1999; Verschoor *et al.*, 1999; Mascola *et al.*, 2000; Nehete *et al.*, 2001; Heeney, 2002). In rhesus macaques, SIVmac251 infection more closely mimics the chronic, progressive T-cell loss that is observed in HIV-1-infected humans, suggesting that other immune mechanisms may be necessary to protect from pathogenic pathways that lead to chronic CD4<sup>+</sup> T-cell loss. When evaluated in this more rigorous SIVmac/rhesus macaque model, the same or similar vaccine strategies have in general not been efficacious (Yasutomi *et al.*, 1995; Nilsson *et al.*, 2001; Allen *et al.*, 2002; Horton

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*et al.*, 2002; Vogel *et al.*, 2002a). Notably, immunization with a NYVAC vector expressing SIV-K6W Env, Gag and Pol recently proved to be partially effective against intravenous challenge with SIVmac251 (Benson *et al.*, 1998). However, a similar effect was not seen in animals that had been challenged via the intrarectal route (Benson *et al.*, 1998). We have recently reported on partial protection against intrarectal challenge with SIVmac251 in cynomolgus monkeys, using a triple-vector vaccine combination that comprised DNA, Semliki Forest virus (SFV) and modified vaccinia virus Ankara (MVA) (Negri *et al.*, 2004). However, cynomolgus monkeys generally develop lower virus loads after challenge with SIVmac251 than do rhesus macaques (ten Haaf *et al.*, 2001). In the current study, we therefore sought to confirm and extend these results in the more stringent SIVmac/rhesus macaque model. As in the previous study, a multi-antigen, multi-vector vaccine strategy was used, i.e. rhesus macaques were immunized sequentially with a combination of three different vectors (DNA, SFV and MVA) that shared the structural Gag, Pol and Env and the regulatory Nef, Rev and Tat antigens derived from SIVmac251. Although all vaccinated animals became infected, their CD4<sup>+</sup> T-cell counts were maintained at pre-infection levels, in contrast to the infected control animals, which all developed a characteristic gradual loss of CD4<sup>+</sup> T cells.

## METHODS

**Animals.** Captive-bred, mature (4- to 5-year-old), outbred Indian rhesus macaques were housed at the Biomedical Primate Research Center, Rijswijk, the Netherlands. The animals were negative for antibodies to SIV-1, simian type D retrovirus and simian T-cell lymphotropic virus. During the course of the study, animals were checked twice daily for appetite and general behaviour and stools were checked for their consistency. Body weight and body temperature were measured every time an animal was sedated for blood collection or immunization. Animals developing opportunistic infections, a body weight loss of >10%, persistently low CD4<sup>+</sup> T-cell counts and high virus loads were considered to be progressing to AIDS and were euthanized. The Institutional Animals Care and Use Committee approved the study protocols according to international ethical and scientific standards and guidelines.

**Vaccines.** For DNA-expression-based vaccines, the following vectors were used: pTH.UbgagPk, pTH.UbpolPk, pTH.UbnefPk, pTH.rev and pTH.tat; all express the *gag*, *pol*, *nef*, *rev* and *tat* genes of SIVmacJ5 under the control of the human cytomegalovirus immediate-early (HCMV IE) enhancer/promoter (Hanke *et al.*, 1998). Vector pND14-G4 contained the SIVmac251 envelope gp120 coding sequence under the control of the HCMV IE enhancer/promoter. All constructs also contained the HCMV intron A sequence 5' of the expressed genes, in order to increase expression from the HCMV enhancer/promoter sequence, and carried the bovine growth hormone (BGH) poly(A) signal/terminator sequence (Rhodes *et al.*, 1994; Hanke *et al.*, 1998). Vector control animals were immunized with empty pTH.

MVA recombinants used in this study expressed the Gag, Pol, Nef, Rev, Tat and envelope gp160 proteins of SIVmacJ5 under control of the vaccinia virus early-late promoter P7.5 (Nilsson *et al.*, 2001; Horton *et al.*, 2002; Vogel *et al.*, 2002b). Vector control animals were immunized with wild-type MVA.

SFV recombinants used in this study expressed the Gag, Pol, Nef, Rev, Tat and envelope gp160 proteins of SIVmacJ5. Genes were expressed in general-expression vectors based on the SFV replicon (Nilsson *et al.*, 2001). Recombinant RNA molecules were transcribed from the inserted genes and subsequently packaged into suicide SFV particles for use as a vaccine. Vector control animals were immunized with SFV-LacZ.

**SIVmac251 virus stock.** Challenge virus stock was prepared from the supernatant of peripheral blood mononuclear cells (PBMCs) from rhesus macaques that were infected with SIVmac251 (kindly provided by Dr Aubertin, Strasbourg, France). Rhesus macaques were inoculated intrarectally by using tenfold dilutions from 10<sup>-1</sup> to 10<sup>-4</sup>, with four rhesus macaques per dilution apart from the 10<sup>-4</sup> dilution, for which three monkeys were used. The resulting number of infected animals in each group was 4, 4, 2 and 0, respectively, giving an intrarectal titre of 10<sup>3</sup> MID<sub>50</sub> ml<sup>-1</sup>.

**Immunization and challenge schedule.** The study comprised three groups of animals. Six animals received the DNA, MVA and SFV vectors expressing the SIV proteins Gag, Pol, Nef, Rev, Tat and Env; four animals received the empty DNA, MVA and SFV vectors (vector controls); two animals were not immunized (naïve controls). Animals received four immunizations at 8-weekly intervals, starting with the DNA vector, followed by MVA, then SFV and finally a second MVA immunization. DNA immunization (100 µg per construct) was performed as a total of six intradermal injections, 100 µl per injection site (100 µg DNA), proxolateral from both inguinal regions. MVA immunization (1 × 10<sup>8</sup> p.f.u. per construct) was given as five intramuscular injections, 500 µl per injection site (1 × 10<sup>8</sup> p.f.u. per site). SFV (1 × 10<sup>8</sup> p.f.u. per construct) was given as two subcutaneous injections, 1000 µl per injection site (3 × 10<sup>8</sup> p.f.u.), proxolateral from both inguinal regions. Eight weeks after the last immunization, all animals were challenged by intrarectal administration of 50 MID<sub>50</sub> of the pathogenic SIVmac251 stock. Fasted monkeys were sedated by ketamine injection and laid on their stomachs with the pelvic region slightly elevated. A feeding tube was inserted 4 cm into the rectum and 3 ml virus diluted 1:60 in sterile, pyrogen-free RPMI 1640 containing 20% inactivated fetal calf serum (FCS) was delivered slowly.

**Cellular immunology assays.** Th cell responses were determined by a standard [<sup>3</sup>H]thymidine incorporation assay, performed 4 weeks after each immunization and after challenge, as described by Verschoor *et al.* (1999). Briefly, PBMCs were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FCS in U-shaped, 96-well microtitre plates at a concentration of 2 × 10<sup>5</sup> cells per well using SIVmac251 rgp130 (NIBSC, EVA655), SIVmac251 rGag (NIH catalogue no. 1845), SIV J5Nef (NIBSC, ARP668) and SIV-Tat (NIBSC, ARP681) (5 µg ml<sup>-1</sup>) to stimulate proliferation. Concanavalin A stimulation (5 µg ml<sup>-1</sup>) was used as a positive control. Cells were incubated for 90 h. During the last 18 h, cells were pulsed with 2.5 µCi [<sup>3</sup>H]thymidine per well. Subsequently, cultures were harvested on glass-fibre filters and label uptake was determined by counting simultaneously in an open-well Packard Matrix counter (direct beta-counter) with 96 counting tubes. Stimulation indices (SIs) were calculated by dividing the mean c.p.m. of antigen-stimulated wells by the mean of the unstimulated wells. An SI of >3.0 was considered to be positive.

Induction of gamma interferon (IFN-γ), interleukin 2 (IL2) and IL4 cytokine responses was measured by using an ELISpot assay, performed 4 weeks after each immunization and after challenge (Verschoor *et al.*, 1999). In brief, 4 × 10<sup>6</sup> PBMCs ml<sup>-1</sup> were cultured in a 24-well tissue culture plate for 24 h in RPMI 1640 medium supplemented with 5% pooled rhesus serum, using SIVmac251 rgp130, SIVmac251 rGag, SIV J5Nef and SIV-Tat (5 µg ml<sup>-1</sup>) to stimulate cytokine production. Phorbol myristate acetate (PMA;

20 ng ml<sup>-1</sup>) plus ionomycin (1 µg ml<sup>-1</sup>) stimulation was used as a positive control. Two weeks after each immunization, IFN-γ ELISpot responses against a panel of peptides that was selected to cover the immunodominant epitopes of SIV Tat, Rev, Nef and Gag were measured. Peptide pools were composed as follows: Tat pool 1 (EVA7069.1–5), Tat pool 2 (EVA7069.6–10), Rev pool 1 (EVA7068.1–4), Rev pool 2 (EVA7068.5–8), Nef pool 1 (EVA7067.1–5), Nef pool 2 (EVA7067.6–10), Nef pool 3 (EVA7067.11–15), Nef pool 4 (EVA7067.16–20), Nef pool 5 (EVA7067.21–25), Nef pool 6 (EVA7067.26–29), Gag pool 1 (EVA7066.1–4), Gag pool 2 (EVA7066.5–8), Gag pool 3 (EVA7066.9–12) and Gag pool 4 (EVA7066.13–16). For enumeration of antigen-specific cytokine production, non-adherent cells were collected and plated at 2 × 10<sup>5</sup> cells per well in a 96-well ELISpot plate with the same antigens added. Microtitre plates were pre-coated with mAbs that were specific for the lymphokine of interest, i.e. anti-IFN-γ mAb MD-1 (Ucytech), anti-IL4 mAb QS-4 (Ucytech) and anti-IL2 mAb B-G5 (Dialclone Laboratories).

Detection of cytokine-secreting cells took place after either 15 h for IL4 or 4 h for IFN-γ and IL2. Cells were lysed and debris was washed away before adding detector antibodies. IFN-γ, IL2 and IL4 were detected by using biotinylated rabbit anti-rhesus IL2, biotinylated rabbit anti-rhesus IFN-γ or biotinylated mouse anti-rhesus IL4. Spots were visualized by using a gold staining/silver enhancement technique (Ucytech). IFN-γ, IL2 or IL4 ELISpot results are expressed as spot-forming cells per 10<sup>6</sup> PBMCs minus background (mean of medium control + 2SD). The assay was discarded if PMA/ionomycin stimulation gave no response.

**Determination of virus load.** A quantitative competitive RNA-PCR was used to estimate the virus load in plasma, as described by ten Haaf *et al.* (1998). For determining the cell-associated virus load, PBMCs or mononuclear cells isolated from peripheral lymph nodes, mesenteric lymph nodes or spleen were cultured in a three- or fourfold dilution range with C8166 indicator cells for 3–6 weeks. PBMCs taken at week 2 after challenge were plated in a range from 250 000 to 250 cells per well by using fourfold dilutions. For the two non-infected macaques (Ri404 and Ri437), 10<sup>7</sup> cells were plated over 24 wells. PBMCs taken at week 31 after challenge were plated in a range from 500 000 to 2000 cells per well by using threefold dilutions. From four macaques with low or undetectable plasma virus loads (Ri404, Ri406, Ri421 and Ri437), 5 × 10<sup>6</sup> PBMCs were plated over 10 wells. Medium was renewed twice a week. Positive cultures were scored via cytopathic effect formation, which was confirmed by RT-PCR.

**Intracellular IFN-γ and IL2 staining.** PBMCs (5 × 10<sup>6</sup> ml<sup>-1</sup>) were incubated at 37 °C for 2 h with anti-CD28 and anti-CD49d antibodies (2 µg each antibody; BD Pharmingen) and either staphylococcal enterotoxin B (1.25 µg ml<sup>-1</sup>; Sigma), pooled peptides (1.25 µg each peptide per sample) or SIV-1 Env protein (1.25 µg ml<sup>-1</sup>). Peptide pools for induction of intracellular cytokines were composed as follows: Tat pool (EVA7069.1–10), Rev pool (EVA7068.1–8), Nef pool (EVA7067.1–29) and Gag pool (EVA7066.1–16).

Cells were treated with brefeldin A (GolgiPlug 1:1000; BD Pharmingen) to inhibit protein trafficking and incubated for 16 h at 37 °C. Cells were then washed with PBS/1% BSA and stained for surface markers by using fluorescein isothiocyanate (FITC)-labelled anti-CD8 (DAKO) and peridinin chlorophyll protein (PerCP)-labelled anti-CD4<sup>+</sup> (clone L200; BD Pharmingen) for 30 min at 4 °C in the dark. Subsequently, cells were washed with PBS/BSA and fixed with cytofix/cytoperm solution (BD Pharmingen) for 20 min at 4 °C. The cells were then washed twice with permeabilization buffer (diluted tenfold in water) and resuspended in permeabilization buffer containing phycoerythrin (PE)-labelled anti-IL2 and allophycocyanin (APC)-labelled anti-IFN-γ mAb. After 30 min incubation at

4 °C, cells were washed twice with permeabilization buffer and fixed in 2% paraformaldehyde in PBS for 16 h. Acquisition was performed on a FACSort flow cytometer collecting 100 000–200 000 lymphocyte-gated events per sample.

**FACS subset analysis.** Macaques were monitored for changes in their T-lymphocyte subsets by flow cytometry analysis, as described by Koopman *et al.* (2001). Briefly, 100 µl EDTA-treated blood was incubated with 10 µl mAb mix in 5 ml polystyrene, round-bottom tubes (Falcon 2058; Becton Dickinson) at room temperature for 15 min. After this incubation, 1.5 ml lysing solution (Becton Dickinson) was added, followed by incubation at room temperature for 10 min and then centrifugation for 10 min at 500 g. The supernatant was aspirated and the cells were resuspended in 5 ml PBS with 1–2% formaldehyde and stored overnight at 4 °C. Flow cytometry was performed on a FACSort using CellQuest software (Becton Dickinson). The following mAbs were used: (a) CD3<sup>FITC</sup>, CD16<sup>PE</sup>, CD8<sup>PerCP</sup>, CD4<sup>APC</sup>; (b) HLA-DR<sup>FITC</sup>, CD20<sup>PE</sup>, CD8<sup>PerCP</sup>, CD4<sup>APC</sup> and (c) CD45RA<sup>FITC</sup>, CD62L<sup>PE</sup>, CD8<sup>PerCP</sup>, CD4<sup>APC</sup>. These mAbs were obtained from BD Pharmingen (CD8<sup>PerCP</sup> clone SK1, CD4<sup>APC</sup> clone SK3, CD62L<sup>PE</sup> clone SK11, HLA-DR<sup>FITC</sup> clone L243, CD20<sup>PE</sup> clone L27, CD3e<sup>FITC</sup> clone SP34, CD16<sup>PE</sup> clone 3G8) or Dialclone (CD45RA<sup>FITC</sup> clone B-C15).

**Humoral responses.** Anti-SIV antibody titres were determined by using a standard whole-SIVmac ELISA. Briefly, serially diluted plasma was assayed in duplicate in microtitre plates that were coated with whole SIVmac lysate. After incubation with anti-human IgG peroxidase conjugate (Sigma)/substrate solution and absorbance measurement, curves were fitted to the data points and used to calculate individual titres, i.e. the dilution at which the curves crossed the cut-off absorbance. Neutralizing antibodies were measured as described by Norley *et al.* (1996). Briefly, serial dilutions of SIVmac251 (six replicates) were incubated with a 1:100 dilution of plasma before addition of C8166 cells. After incubation for 7 days, wells were tested for SIVmac Gag p27 by using an antigen-capture ELISA and the virus titres (TCID<sub>50</sub>) determined. Yield reduction for each sample was calculated as the titre of virus in the absence of plasma divided by the titre in the presence of plasma.

**Statistical analysis.** An unpaired *t*-test was used to compare virus load between the group of six vaccinated animals and the four control animals that became infected. For reasons explained in Results, the two non-infected animals were excluded from the analysis. Statistical analysis was performed for peak virus load, measured at week 2 after infection, and steady-state plasma virus load, measured at week 10 after infection. In order to study the effect of vaccination on the persistence of CD4<sup>+</sup> T cells, the change in CD4<sup>+</sup> T-cell count observed between the time before infection and week 31 after infection was calculated in the group of vaccinated animals, as well as in the group of control animals that became infected. Both within the vaccine group and within the control group, statistical significance of the change in CD4<sup>+</sup> T-cell count was calculated by using Student's paired *t*-test. An unpaired *t*-test was used to compare anti-SIV antibody levels and SIV-neutralizing antibody levels, measured at week 20 after infection, between the group of six vaccinated animals and the four control animals that became infected.

## RESULTS

### Vaccine-induced, SIV-specific immune responses

Four weeks after each immunization, T-cell proliferative responses, as well as IFN-γ, IL2 and IL4 cytokine responses against SIV Env, Nef, Gag and Tat proteins, were

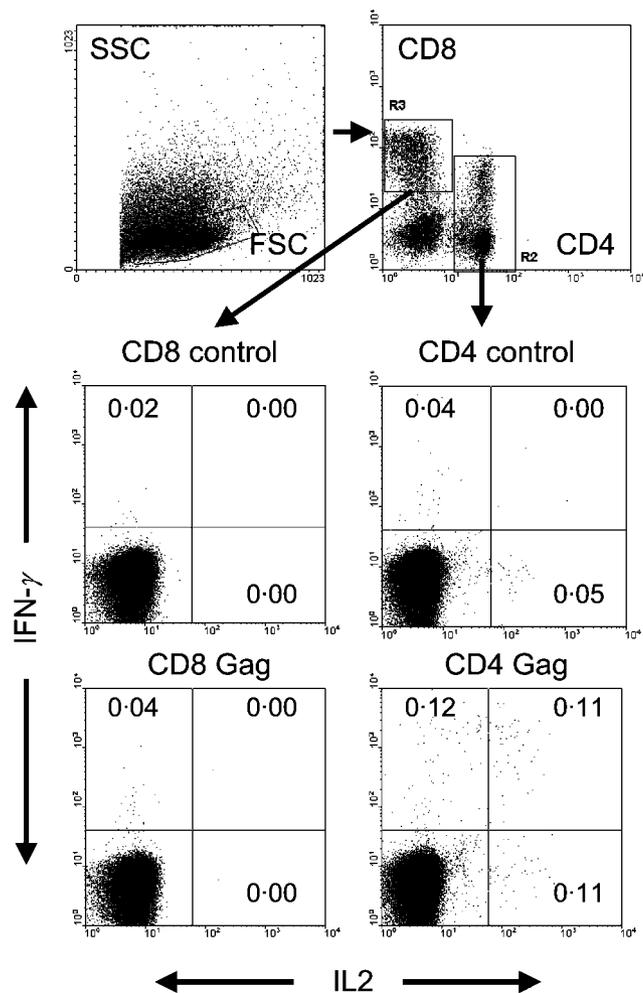
**Table 1.** Antigen-specific T-cell cytokine responses and lymphoproliferation measured 4 weeks after the last immunization

Values in bold indicate strong responses: for the ELISpot assay, this was defined as  $>40$  spot-forming cells per  $10^6$  PBMCs; for the lymphoproliferation assay, as  $SI > 3.0$ . Mean ELISpot background responses were  $115 \pm 90$  for IFN- $\gamma$ ,  $15 \pm 13$  for IL2 and  $18 \pm 20$  for IL4. ND, Not determined.

Group/macaque	IFN- $\gamma$ ELISpot*				IL2 ELISpot*				IL4 ELISpot*				Lymphoproliferation†			
	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat
Vaccine:																
Ri406	30	10	0	10	5	0	10	0	0	0	10	20	1.2	0.7	2.0	1.4
Ri408	0	0	0	0	25	35	15	0	35	75	85	85	3	1.5	3.3	2.6
Ri418	0	0	0	0	0	5	0	0	0	20	0	90	1.6	1.1	3	1.3
Ri420	400	0	400	0	95	0	170	0	160	10	265	15	3.2	1.2	4.3	1.9
Ri445	40	0	210	0	35	15	45	0	25	15	40	15	2.7	2.2	3	2.4
Ri447	0	0	180	0	20	25	55	0	0	0	20	0	1.5	1.4	2.5	1.3
Vector control:																
Ri403	0	0	0	0	5	0	20	0	0	0	0	20	1.2	0.9	2.5	1.6
Ri404	0	0	0	0	5	0	0	0	0	0	0	0	1.3	0.8	1.8	1.7
Ri411	0	0	0	0	0	0	0	0	30	15	5	0	1.1	0.7	1.6	1.2
Ri437	0	0	0	ND	0	0	0	ND	0	0	0	ND	0.9	1.1	1.1	ND

\*Spot-forming cells per  $10^6$  PBMCs minus background (mean of medium control + 2SD).

†SI (mean c.p.m. in antigen-stimulated wells/mean c.p.m. in unstimulated wells).



determined. DNA priming and the first MVA booster immunization did not give any measurable responses (data not shown). Some positive responses were observed after the SFV booster immunization (not shown) and a further increase was seen following the final MVA immunization (Table 1). IFN- $\gamma$  production was observed against SIV Gag in three of the six immunized macaques (Ri420, Ri445 and Ri447) and one of these three animals (Ri420) also had a response against SIV Env. The same animals showed IL2 production against the same proteins (Table 1). In contrast, IL4 production was seen in only one of these three macaques (Ri420), against SIV Env and Gag, but not in the two other animals, reflecting a Th1-biased response in these animals. Importantly, IL4 production was also observed in two other macaques (Ri408 and Ri418) that had no IFN- $\gamma$  response. Both animals recognized SIV Tat and Ri408 also responded to the other SIV proteins. On the basis of these cytokine responses, macaques could be subdivided into Th1-type responders (Ri445 and Ri447), Th2 responders (Ri408 and Ri418), a Th0 responder (Ri420) and a non-responder (Ri406). Four of the six immunized animals also had some lymphoproliferative responses,

**Fig. 1.** Example of intracellular cytokine response after stimulation with Gag peptides as seen in macaque Ri447, 2 weeks after the last MVA immunization. PBMCs from macaque Ri447 were either stimulated with a Gag peptide pool or cultured without peptides (control). Subsequently, they were stained for CD4, CD8, IFN- $\gamma$  and IL2. Cells within the lymphogate were first plotted for CD4 against CD8 and then IFN- $\gamma$  was plotted against IL2 for both the CD4<sup>+</sup> CD8<sup>-</sup> and the CD4<sup>-</sup> CD8<sup>+</sup> cell populations.

although this was barely above the background level (SI > 3). No responses were detected in animals that had been immunized with control vectors.

Two weeks after each immunization, IFN- $\gamma$  ELISpot responses were measured against several SIV Tat, Rev, Nef and Gag peptide pools. After the last MVA immunization, clear responses were detected in macaques Ri420, Ri445 and Ri447 only (data not shown), which were the same animals that produced IFN- $\gamma$  in response to the proteins. Responses were mostly directed against Rev, Nef and Gag peptides (data not shown).

In order to establish whether CD4<sup>+</sup> Th1 cells or CD8<sup>+</sup> cytotoxic cells were responsible for the IFN- $\gamma$  production seen in the ELISpot assay, an intracellular cytokine assay was performed (example shown in Fig. 1). As shown in Table 2, in agreement with the ELISpot results, Gag-specific IFN- $\gamma$  production was seen in three of the six vaccinated macaques (Ri420, Ri445 and Ri447). Importantly, only CD4<sup>+</sup> cells produced IFN- $\gamma$ . In contrast to the ELISpot data, no response was seen against SIV Env. However, as no peptides were available for Env, we used the protein in this assay and this may have been less optimal than the peptides. Strikingly, macaque Ri408 showed Nef-specific IFN- $\gamma$  production, both by CD4<sup>+</sup> and by CD8<sup>+</sup> T-cells (Table 2). Although such a response was not seen in the ELISpot assay on this macaque (Table 1), it must be stated that it gave a high background, which may have precluded detection. None of the control animals showed a response. The proportion of positive cells in the medium control was below 0.05 %.

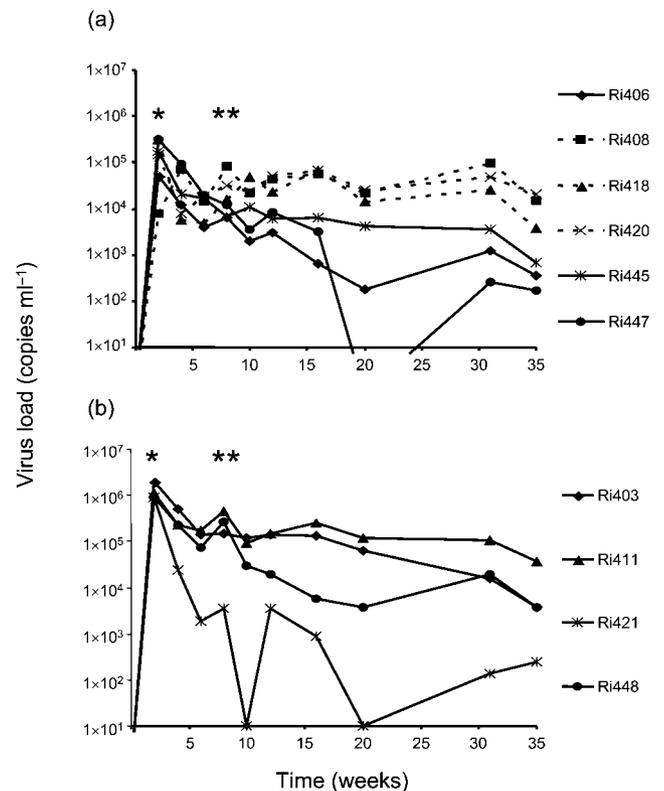
**Table 2.** SIV-specific intracellular IFN- $\gamma$  production in CD4<sup>+</sup> and CD8<sup>+</sup> T cells measured 2–4 weeks after the last MVA immunization

Percentage IFN- $\gamma$  expression in stimulated cultures minus the background response seen in unstimulated cultures is shown. Background responses were always below 0.05 %. Responses were considered to be positive (shown in bold) when greater than twice the background.

Macaque	CD4 <sup>+</sup> T cells				CD8 <sup>+</sup> T cells			
	Env	Gag	Nef	Tat	Env	Gag	Nef	Tat
Ri406	0.05	0.05	0.03	0.03	0.00	0.00	0.00	0.00
Ri408	0.00	0.03	<b>0.11</b>	0.03	0.04	0.06	<b>0.19</b>	0.06
Ri418	0.02	0.05	0.03	0.01	0.00	0.01	0.01	0.02
Ri420	0.03	<b>0.07</b>	0.05	0.02	0.00	0.00	0.00	0.00
Ri445	0.00	<b>0.07</b>	0.04	0.01	0.00	0.02	0.03	0.03
Ri447	0.00	<b>0.19</b>	0.02	0.01	0.00	0.02	0.01	0.01
Ri403	0.02	0.02	0.02	0.01	0.03	0.02	0.06	0.01
Ri404	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Ri411	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Ri437	0.00	0.00	0.01	0.00	0.02	0.00	0.02	0.02

**Challenge results**

Eight weeks after the last immunization, all animals were challenged by intrarectal administration of SIVmac251. All six immunized animals and the two naïve-control animals became infected, as determined by RT-PCR analysis of plasma (Fig. 2). However, only two of four vector-control animals became virus-positive (Fig. 2). The same findings were obtained when the cell-associated virus load was measured (data not shown). In addition, PBMCs from the two RT-PCR-negative animals were also SIV DNA-negative (data not shown). Intrarectal challenge is known to be more susceptible to variation than intravenous challenge and animals often remain negative for reasons that are probably associated with the mode of application. If only



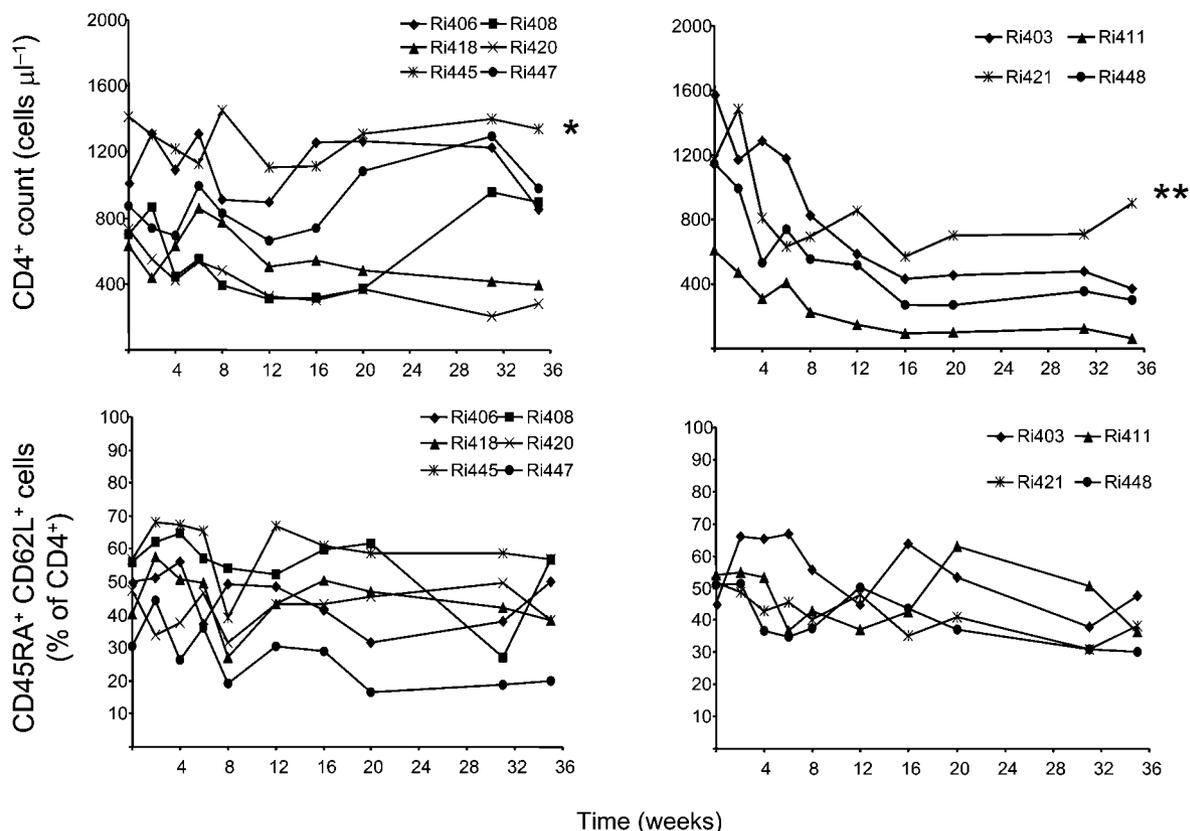
**Fig. 2.** Plasma viral RNA levels of individual macaques after intrarectal challenge with SIVmac. Plasma virus loads in (a) vaccinated macaques (Ri406, Ri408, Ri418, Ri420, Ri445 and Ri447) and (b) macaques that received control vectors (Ri403 and Ri411) and naïve animals (Ri421 and Ri448) that became virus-positive are shown. Two other control animals (Ri404 and Ri437) remained virus-negative (data not shown). Dashed lines are used to discriminate animals with an IL4 response in the ELISpot assay (Table 1). \*Difference in peak virus levels between vaccine and control groups is statistically significant ( $P < 0.01$ ); \*\*difference in steady-state virus levels between vaccine and control groups is not statistically significant.

the infected animals were considered, then the immunized animals had a somewhat lower viraemia peak (Fig. 2) than the control group ( $P < 0.01$ , unpaired *t*-test). However, no significant difference was seen between the groups in steady-state virus levels that were obtained 10 weeks after challenge (unpaired *t*-test). Indeed, steady-state virus levels did vary considerably, both in the vaccine group (low in three of six animals) and in the control group (low in macaque Ri421). Importantly, all infected control-group animals showed a gradual decrease in CD4<sup>+</sup> T-cell count ( $P < 0.05$ , paired *t*-test), although in macaque Ri421 (which had a low virus load), the decrease was less pronounced than in the other animals. In contrast, in the vaccine group, the CD4<sup>+</sup> T-cell count was not changed significantly (paired *t*-test) (Fig. 3). No differences between the groups were seen with regard to the percentage of CD45RA<sup>+</sup> CD62L<sup>+</sup> naïve CD4<sup>+</sup> T cells, indicating that there was no change in CD4<sup>+</sup> T-cell memory-naïve subset composition (Fig. 3).

### SIV-specific immune responses after challenge

Four weeks after challenge, T-cell proliferative responses, as well as IFN- $\gamma$ , IL2 and IL4 cytokine responses against SIV Env, Nef, Gag and Tat proteins, were determined. For IFN- $\gamma$ , there was a high background response of  $175 \pm 136$  spots per  $10^6$  PBMCs (Table 3). This made it difficult to draw conclusions, although SIV Gag-specific IFN- $\gamma$  production was observed in two macaques (Ri406 and Ri420). Strikingly, no IL2 production and almost no IL4 production was detected, implying that these Th responses were lowered after infection. In contrast, strong proliferative responses (SI > 9) were seen in three of the four infected control animals, whereas the immunized animals had only low responses (SI > 3) (Table 3).

SIV-specific antibodies in serum, as well as SIV-neutralizing antibodies, were determined after the last MVA booster immunization, as well as at several time points after challenge (Fig. 4). No anti-SIV antibodies could be detected



**Fig. 3.** CD4<sup>+</sup> T-cell counts (top) and number of CD4<sup>+</sup> naïve T cells (bottom) in individuals after intrarectal challenge with SIVmac. Absolute CD4<sup>+</sup> T-cell counts in the group of vaccinated animals (top left) and control animals (top right), as well as the number of naïve CD4<sup>+</sup> T cells, defined as CD4<sup>+</sup> CD45RA<sup>+</sup> CD62L<sup>+</sup>, in the group of vaccinated animals (bottom left) and control animals (bottom right) are shown. The two control animals that did not become infected and are therefore not expected to lose CD4<sup>+</sup> T cells are not shown. \*Change in CD4<sup>+</sup> T-cell count in vaccine group between weeks 0 and 31 is not statistically significant. \*\*Change in CD4<sup>+</sup> T-cell count in control group between weeks 0 and 31 is statistically significant ( $P < 0.05$ ).

**Table 3.** Antigen-specific T-cell cytokine and lymphoproliferative responses 4 weeks after challenge with SIVmac251

Values in bold indicate strong responses: for the ELISpot assay, this was defined as >40 spot-forming cells per 10<sup>6</sup> PBMCs; for the lymphoproliferation assay, as SI > 3.0. Mean ELISpot background responses were 175 ± 136 for IFN- $\gamma$ , 15 ± 10 for IL2 and 15 ± 4 for IL4.

Group/maquette	IFN- $\gamma$ ELISpot*				IL2 ELISpot*				IL4 ELISpot*				Lymphoproliferation†			
	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat	Env	Nef	Gag	Tat
Vaccine:																
Ri406	0	0	<b>250</b>	<b>85</b>	0	0	0	0	0	0	0	40	1.7	1.2	2.9	1.5
Ri408	0	0	0	0	0	0	0	0	5	0	25	0	2	0.9	2.7	1.1
Ri418	40	0	0	0	0	0	0	0	0	0	0	0	1.7	1.2	<b>3.7</b>	<b>3.1</b>
Ri420	35	0	<b>350</b>	10	0	0	0	0	0	0	0	20	<b>3.6</b>	1.3	<b>5.1</b>	<b>5.4</b>
Ri445	0	0	0	0	15	0	0	25	0	0	0	0	2.2	1.3	1.9	2.3
Ri447	0	0	0	0	0	0	0	10	0	0	0	5	1.3	1.2	3.8	2
Vector control:																
Ri403	0	0	20	0	0	0	0	0	0	0	0	0	2.3	1.1	<b>9.4</b>	2.9
Ri404	0	0	0	0	0	0	0	0	0	0	0	0	1.3	0.6	1.2	1.3
Ri411	0	0	0	0	0	0	0	0	0	0	0	0	1.4	0.9	<b>3.2</b>	2.4
Ri437	0	0	0	0	0	0	0	0	0	0	0	0	1.2	0.8	1.8	2.8
Naïve control:																
Ri421	0	0	0	0	0	0	0	0	0	0	0	0	<b>5.3</b>	1.1	<b>11.6</b>	<b>5</b>
Ri448	0	0	0	0	5	0	0	0	0	0	0	0	2.5	1	<b>9.9</b>	<b>9.6</b>

\*Spot-forming cells per 10<sup>6</sup> PBMCs minus background (mean of medium control + 2SD).

†SI (mean c.p.m. in antigen-stimulated wells/mean c.p.m. in unstimulated wells).

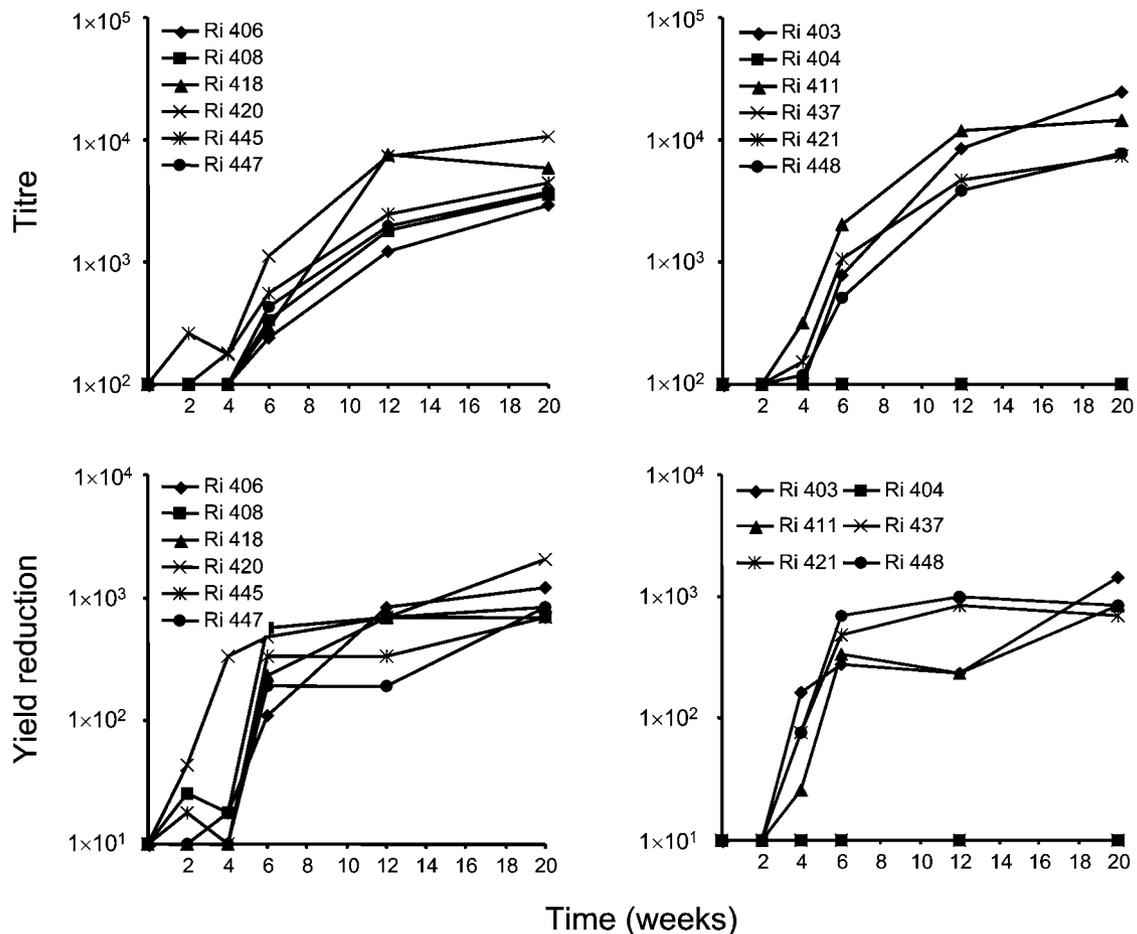
before challenge (Fig. 4). However, 4–6 weeks after challenge, all the vaccinated animals, as well as the control animals that became SIV-infected, developed antibodies that could neutralize SIV infection (Fig. 4). No significant difference in anti-SIV or SIV-neutralizing antibody titres was seen between the vaccine group and the infected control group (unpaired *t*-test). The two virus-negative animals also remained seronegative.

## DISCUSSION

Due to the difficulty in generating neutralizing antibodies against HIV, as well as against SIVmac251 and SIVmac239, there has been strong interest in improving vaccine-induced cellular immune responses to combat and, if possible, to eradicate viral infection in the very early stages (Montefiori *et al.*, 1996; Langlois *et al.*, 1998; Letvin *et al.*, 2002). Earlier studies concentrated on testing different single vectors for delivery of HIV or SIV proteins but, more recently, the focus has shifted to combining vectors in so-called prime/boost strategies to enhance antigen-specific responses and to reduce the possible impact of anti-vector responses (Robinson *et al.*, 1999; Allen *et al.*, 2000; Amara *et al.*, 2001; Nilsson *et al.*, 2001; Stittelaar *et al.*, 2002; Casimiro *et al.*, 2003). In this study, we chose to take this one step further by combining three different strategies, i.e. DNA priming plus SFV and MVA booster immunizations. This strategy of combining different vectors was chosen because anti-vector responses are known to occur and to inhibit induction of HIV-specific immune responses (Sharpe *et al.*, 2001).

In our study, we found HIV-specific immune responses in five of six immunized macaques. In three of these, strong, antigen-specific IL4 production was observed with additional antigen-specific IFN- $\gamma$  production in one of the three (Th0). The other two animals were typical Th1 responders with high IFN- $\gamma$  and low IL4 production. The strength of the immune responses that were induced with this triple-vaccination strategy was, for the lymphoproliferative responses, comparable with previous DNA prime/one-viral-vector boost strategies. The intracellular cytokine responses were slightly lower in comparison with other studies (Allen *et al.*, 2000, 2002; Nilsson *et al.*, 2001; Horton *et al.*, 2002; Vogel *et al.*, 2002b). However, a direct comparison is often difficult because of differences in assays used, as well as inclusion of Mamu A\*01-positive macaques with high Gag181–189- and Tat28–35-specific responses (Allen *et al.*, 2000, 2002; Horton *et al.*, 2002; Vogel *et al.*, 2002b). Although none of the vaccinated animals was protected against infection with the challenge virus, a reduction in peak viraemia was seen and, more importantly, CD4<sup>+</sup> T cells were relatively well-preserved.

In this study, vaccine efficacy was tested by intrarectal challenge with the highly pathogenic SIVmac251. This model is thought to be highly relevant to HIV infection in humans because: (i) most humans get infected via mucosal exposure; (ii) as with HIV-1 in humans, it typically leads to a gradual depletion of CD4<sup>+</sup> T cells, followed by the onset of opportunistic infections and development of AIDS; and (iii) as with HIV, it is difficult to raise neutralizing



**Fig. 4.** Antibody responses and neutralizing antibody responses in individual animals after intrarectal challenge with SIVmac. Antibody responses in serum to whole SIVmac lysate from the group of vaccinated animals (top left) and control animals (top right) are shown. Neutralizing antibody responses in the sera of vaccinated animals (bottom left) and control animals (bottom right) are shown as the reduction in SIVmac251 titre in C8166 cells in the presence of a 1 : 100 serum dilution.

antibodies to the virus. All six vaccinated animals became infected, as well as two of the four vector control animals and both the untreated, naïve-control animals, as determined by quantitative RT-PCR performed on plasma samples. These results were confirmed by DNA PCR on PBMCs, as well as a by co-culture of PBMCs with C8166 indicator cells. In order to confirm the virus-negative status of the two PCR-negative animals, DNA PCR and co-culture assays were also performed on peripheral lymph nodes, mesenteric lymph nodes and spleen mononuclear cells that were obtained at autopsy. In addition, no cellular or humoral responses were found in these animals (Table 3 and Fig. 4). With mucosal challenge, the virus needs to cross several barriers in order to reach the immune cells that are susceptible to infection. In addition, the innate immune system may provide ways to neutralize the virus via soluble factors as well, e.g. the activity of natural killer cells. These factors, which are still far from understood, may be the reason that mucosal challenges are generally far less reliable than an intravenously applied virus challenge.

The virus stock used in this study has been tested in several other studies, where 25 of 31 control animals became infected, underscoring the fact that failed challenges do occur.

If the two virus-negative animals were considered to represent 'failed challenges' and only the infected animals were compared, then the group of vaccinated animals showed a somewhat lower peak virus level in plasma 2 weeks after challenge than the control group. However, at later time points when steady-state virus levels were reached, plasma virus levels in vaccinated, as well as control, animals varied between 500 and 300 000 copies ml<sup>-1</sup> and no clear differences were seen. Strikingly, the three animals with an IL4 response were less effective at controlling virus load (Table 1, Fig. 2). The two animals with a typical Th1-type response were among the three animals that had a relatively low steady-state plasma virus load. This would be in agreement with experiments in mice, where both the IFN- $\gamma$  produced by Th cells and the Th

cell-mediated induction of CTL responses were shown to be important in suppression of virus replication. Importantly, the numbers of CD4<sup>+</sup> T cells were well-maintained in the vaccinated animals (Fig. 3), whereas all four control animals that became infected showed a gradual loss of CD4<sup>+</sup> T cells. In contrast to previous reports (Veazey *et al.*, 2000; Koopman *et al.*, 2001), we did not observe an increase in the number of CD45RA<sup>+</sup> CD62L<sup>+</sup> naïve CD4<sup>+</sup> T cells in peripheral blood after SIVmac infection (Fig. 3). It is possible that differences in the viral isolate being used may play a role. During the course of the study, none of the animals developed AIDS symptoms and CD4<sup>+</sup> T-cell counts remained relatively stable from weeks 16 to 32 after infection. However, in several animals, a small further decrease in CD4<sup>+</sup> T-cell count was seen at the last time point, i.e. week 36, and disease progression may be envisaged on a longer time-course. Also, one could speculate about possible disease progression in two macaques, Ri420 and Ri418 from the vaccine group, which had a relatively low CD4<sup>+</sup> T-cell count. However, macaque Ri408 from this vaccine group also started with a relatively low CD4<sup>+</sup> T-cell count, but at later time points, its CD4<sup>+</sup> cell numbers increased (Fig. 3).

After challenge, there was a strong reduction in IL2 and IL4 production (Table 3). This may indicate that, as a result of acute infection, responses were shifted to IFN- $\gamma$  production. However, the high background of this assay made it difficult to confirm this supposition. A similarly high background after challenge was observed in the intracellular cytokine assay (data not shown). Surprisingly, high lymphoproliferative responses were seen after challenge in the non-vaccinated control animals that became infected. As it was shown recently that HIV-specific CD4<sup>+</sup> T cells are particularly prone to HIV infection (Douek *et al.*, 2002), one could speculate that these high proliferative responses may have contributed to the decline of CD4<sup>+</sup> T cells.

As expected, the vaccines used in this study did not induce antibodies against the virus. However, all infected animals did develop high antibody titres that were able to block SIV infection *in vitro* (Fig. 4). Apparently, these neutralizing antibodies are not effective in the animals as high virus loads were seen, despite the presence of these antibodies. Lack of correlation between the post-challenge levels of neutralizing antibodies in plasma and suppression of virus load is not unusual. In a study by Vogel *et al.* (2002a), animals with the lowest neutralizing antibody levels during chronic infection also had the lowest plasma virus loads. This may reflect the artificial nature of the *in vitro* assays that were used to measure such antibodies (T cell-adapted SIVmac and human T-cell indicator cells) or may reflect a genuine failure of neutralizing antibodies to significantly influence virus levels in infected animals.

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