

# Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV<sub>251</sub> infection of macaques

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Prolonged antiretroviral therapy (ART) is not likely to eradicate human immunodeficiency virus type I (HIV-I) infection. Here we explore the effect of therapeutic immunization in the context of ART during primary infection using the simian immunodeficiency virus (SIV<sub>251</sub>) macaque model. Vaccination of rhesus macaques with the highly attenuated poxvirus-based NYVAC-SIV vaccine expressing structural genes elicited vigorous virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in macaques that responded effectively to ART. Following discontinuation of a six-month ART regimen, viral rebound occurred in most animals, but was transient in six of eight vaccinated animals. Viral rebound was also transient in four of seven mock-vaccinated control animals. These data establish the importance of antiretroviral treatment during primary infection and demonstrate that virus-specific immune responses in the infected host can be expanded by therapeutic immunization.

## Introduction

The introduction of highly active antiretroviral therapy (HAART), a combination of reverse transcriptase and protease inhibitors, has decreased morbidity and mortality in patients infected with HIV-I (ref. 1). Complex dosing schedules and undesirable side effects, however, frequently result in incomplete adherence and possibly emergence of drug-resistant viral strains.

HIV-I eradication by prolonged HAART treatment appears to be unlikely because of the persistence of a cellular reservoir of infectious HIV-I (refs 2,3). Discontinuation of HAART, in fact, results in viral rebound in most individuals chronically infected with HIV-I (ref. 4). In some patients, intermittent HAART use resulted in temporary containment of HIV-I replication<sup>5,6</sup>. In one of these studies, virus suppression correlated with the strength and breadth of virus-specific CD8<sup>+</sup> T cell response, affirming the importance of cytotoxic response in the control of HIV-I replication<sup>7-11</sup>. Most of the patients with contained viremia were treated during primary infection, suggesting that early HAART treatment may limit immunological damage, as demonstrated by the preservation of CD8<sup>+</sup> T cells and the CD4<sup>+</sup> t cell response<sup>12-14</sup>.

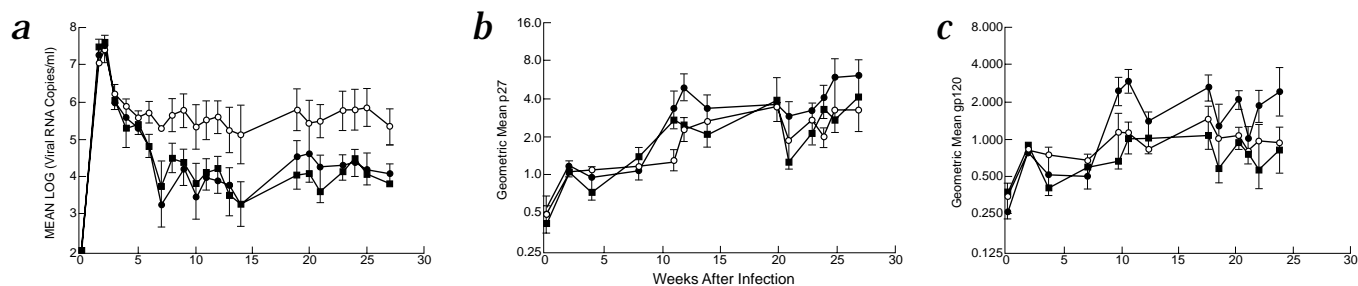
The fact that vigorous host immune response correlates with containment of HIV-I replication and the recent appreciation that prolonged HAART treatment results in a progressive decline in virus-specific response<sup>15-17</sup> provide the rationale to use im-

mune-based strategies to further strengthen the host response to the virus. Here we designed a study in the SIV<sub>251</sub> rhesus macaque model to assess whether early ART intervention in primary SIV<sub>251</sub> infection per se results in restoration of virus-specific immune responses sufficient to contain viremia following ART discontinuation; whether vaccination of macaques enhances SIV-specific CD4<sup>+</sup> T-helper and CD8<sup>+</sup> t cell cytotoxic responses in ART-treated and untreated macaques; and whether the immune responses induced by vaccination correlated with the ability of the host to control viremia following ART suspension.

As a vaccine modality, we have chosen, among the several poxvirus-based vaccine candidates available<sup>18-21</sup>, the highly attenuated poxvirus NYVAC-SIV-*gag-pol-env* (NYVAC-SIV-*gpe*) live recombinant vaccine candidate because of its ability to induce both CD4<sup>+</sup> and CD8<sup>+</sup> t cell responses in rhesus macaques and because of its demonstrated effectiveness as a preventive vaccine candidate<sup>18,22</sup>.

## Results

We inoculated 24 macaques intravenously with ten infectious doses of highly pathogenic SIV<sub>251</sub> (R. Pal *et al.*, manuscript in preparation). The animals were divided into three groups of eight animals each (A, B and C). Animals in groups A and B, but not C, received ART. Animals in groups B and C also received three intramuscular immunizations with NYVAC-SIV-*gpe* (10<sup>8</sup>



**Fig. 1** Statistical analysis of the plasma virus load and proliferative response to p27 Gag and gp120 in the immunized ART-treated macaques from groups A (■), B (●) and C (○). **a**, The mean of the log plasma viremia for all animals in each group is graphically presented up to week 27. The Wilcoxon rank sum test was applied to the mean of the log of plasma viral RNA. The differences between groups A and C were significant at  $P = 0.02$  and between groups B

and C at  $P = 0.028$  within the first 8 weeks of treatment and remained significant thereafter. Graphical representation of the geometric mean value for p27 Gag **b**, and gp120 **c**, for all animals in each group up to week 27. Differences among groups were evaluated using repeated measures ANOVA of the log of the SI over multiple times. The levels of statistical significance of these immune responses after each immunization are summarized in Table 1.

plaque forming unit each), whereas animals in group A received the mock vaccine (NYVAC) at the same time and doses. Each group included 3 Mamu-A\*01 (*Macaca mulata*; nomenclature for rhesus MHC; refs. 23,24) animals. Most Mamu-A\*01 animals infected by SIV recognize the immunodominant Gag peptide p11C, C→M (hereafter referred to as peptide 181; refs. 25,26) within the SIV Gag protein<sup>27</sup>, allowing for the quantitative analysis of CD8<sup>+</sup> response with a tetramer formed by 4 Mamu-A\*01 molecules and peptide 181. By 2 weeks after infection, all animals developed acute viremia with peak viral loads ranging between  $10^7$  and  $10^9$  viral RNA copies per milliliter of plasma (Fig. 1a). All 24 macaques seroconverted to SIV<sub>251</sub> Gag, Pol and Env antigens by week 8 (data not shown), but failed to develop lymphoproliferative response (LPR) to SIV<sub>251</sub> p27 Gag or Env proteins during the first 10 weeks postinfection (Fig. 1b, c).

Gag-specific CD8<sup>+</sup> T cell responses developed in all Mamu-A\*01 animals as measured by the Mamu-A\*01-p11C, C→M tetramer (Gag 181 tetramer), peaking at weeks 2 and 4 postinfection (range 0.55–3.1% of total CD8<sup>+</sup> T cells in blood), consistent with previous observations<sup>28</sup>. These CD8<sup>+</sup> T cells could be expanded following *in vitro* stimulation with the Gag 181 peptide for 7 days and the peak values of cells binding Gag-181 ranged between 4.5 and 67% of total CD8<sup>+</sup> cells. The specificity of tetramer staining was demonstrated in parallel experiments using fresh and cultured peripheral blood mononuclear cells (PBMC) from Mamu-A\*01-positive naive animals and SIV<sub>251</sub>-infected non-Mamu-A\*01 animals (data not shown).

On day 15 after SIV<sub>251</sub> infection, animals in groups A and B were started on ART. Within the 25 weeks of treatment, ART reduced plasma viremia in animals from both groups A and B compared with untreated group C (Fig. 1a). As expected, no significant difference in viremia was observed between groups A and B (Fig. 1a).

At 10, 19 and 23 weeks after infection, all macaques in groups B (ART-treated) and C (untreated) were immunized intramuscularly with  $10^8$  pfu of NYVAC-SIV-*gpe* vaccine, whereas animals in group A (ART-treated) received  $10^8$  pfu of mock-NYVAC parental virus.

Immunizations with NYVAC-SIV-*gpe* increased LPR to both p27 Gag and gp120 Env antigens in macaques from group B, but not

in the viremic animals from group C (Fig. 1b, c and Table 1).

As expected, mock-vaccinated, ART-treated macaques from group A had lower LPR to both p27 Gag and gp120 than the animals vaccinated with NYVAC-SIV-*gpe* in group B, and the differences between the two groups were significant after the first and second immunizations (Table 1). The low level of LPR observed in animals from group C suggest that the ability of the NYVAC-SIV-*gpe* vaccine to induce measurable CD4<sup>+</sup> T-helper response was dependent on viremia suppression by ART treatment. To confirm this, we calculated the correlation coefficient from the values of LPR and virus load after each immunization. LPR to both viral antigens were detectable at levels of viremia below  $10^5$  copies/ml (Fig. 2).

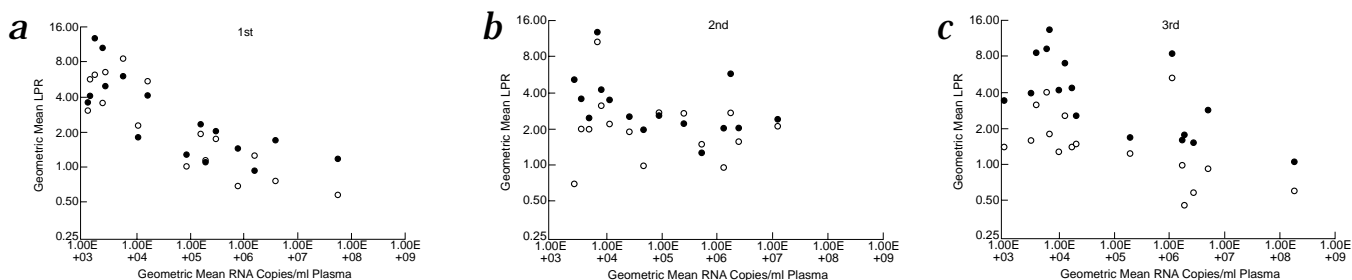
As an indicator for CD8<sup>+</sup> T cell responses induced by NYVAC-SIV, we measured Gag 181-tetramer-staining cells following NYVAC-SIV-*gpe* vaccination or mock-NYVAC vaccination in fresh and cultured PBMC of all Mamu-A\*01 animals. Following the second and third immunizations with NYVAC-SIV-*gpe* vaccine (7 measurements during weeks 19–27), an expansion of CD3<sup>+</sup> CD8<sup>+</sup> T cells staining with Gag 181-tetramer was observed in the PBMC of animals in group B treated with ART, but not in the animals of group C, or in the mock-NYVAC-vaccinated animals from group A (Fig. 3a).

The kinetics of induction of virus-specific fresh or cultured Gag 181-tetramer-positive CD8<sup>+</sup> T cells for each Mamu-A\*01 animal is shown (Fig. 3b, c). To assess whether the Gag 181 CD8<sup>+</sup> T cells had *ex vivo* effector cytotoxic T-lymphocyte (CTL) function, we measured bulk cytolytic activity in the PBMC obtained at week 23 from Mamu-A\*01 animals using autologous transformed B cells pulsed with the Gag 181 peptide as a target.

**Table 1** Statistical analysis of LPR to p27 Gag and gp120 following the three immunizations

Animal group	1 <sup>st</sup> immunization (weeks 11, 12, 14)*	2 <sup>nd</sup> immunization (weeks 20, 21, 23)*	3 <sup>rd</sup> immunization (weeks 24, 25, 27)*	Overall period, weeks 11–27*
B versus C				
p27	$P = 0.021$	$P = 0.28$	$P = 0.024$	$P = 0.026$
gp120	$P = 0.012$	$P = 0.029$	$P = 0.21$	$P = 0.004$
B versus A				
p27	$P = 0.039$	$P = 0.039$	$P = 0.086$	$P = 0.0097$
gp120	$P = 0.014$	$P = 0.0095$	$P = 0.18$	$P = 0.0002$

\*Time points of statistical analysis.



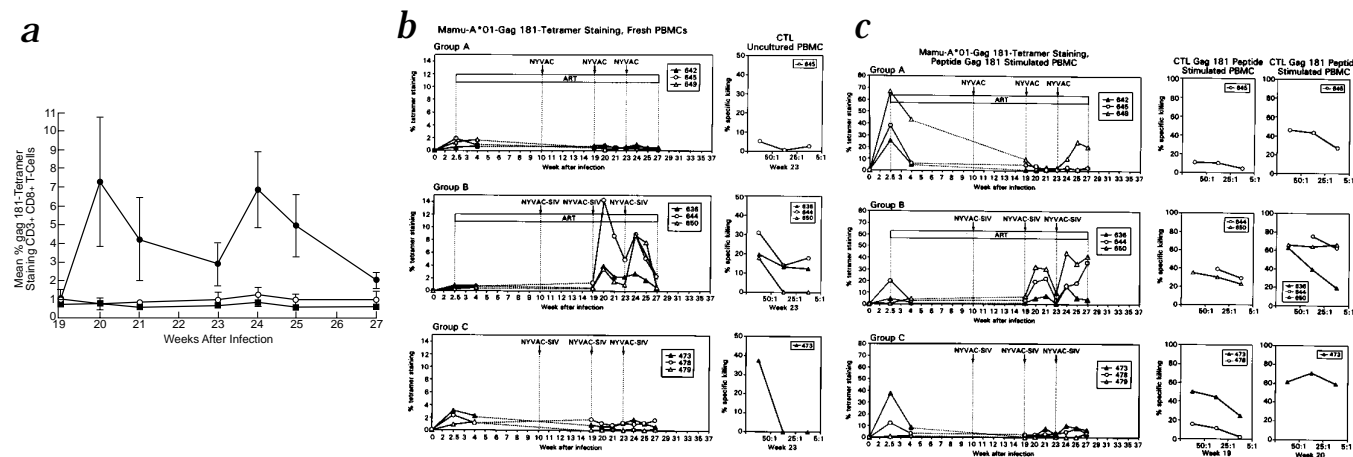
**Fig. 2** Inverse correlation between LPR and viremia. In this analysis, only animals from groups B and C were included because they all received NYVAC-SIV-*gpe* vaccine. Each symbol corresponds to the geometric mean of LPR to p27 Gag (●) or gp120 (○) on the y axis and to the geometric mean of virus load (x axis) at the first and second weeks following each im-

munization. The Spearman rank correlation coefficients, adjusted for the mean by group, were as follows: **a**, first immunization, p27 = -0.68 ( $P = 0.0038$ ), gp120 = -0.75 ( $P = 0.0008$ ); **b**, second immunization, p27 = -0.42 ( $P = 0.12$ ), gp120 = +0.01 ( $P = 0.96$ ); **c**, third immunization, p27 = -0.51 ( $P = 0.041$ ), gp120 = -0.56 ( $P = 0.025$ ).

Significant CTL activity was observed in nonstimulated PBMC populations of animals 636 and 644 in group B, but in none of the animals from groups A and C (Fig. 3b, right). To further confirm the specificity of the Gag 181-staining CD8<sup>+</sup> T cells, the PBMC from the Mamu-A\*01 animals collected at the time points indicated (Fig. 3b) were stimulated *in vitro* with Gag 181 peptide and their ability to bind to the Mamu-A\*01 Gag 181 tetramer assessed. CD3<sup>+</sup> CD8<sup>+</sup> T cells binding Gag 181-tetramer were expanded *in vitro* at most time points and the extent of this expansion was significantly greater in samples from animals in group B than in group C following NYVAC-SIV-*gpe* vaccination ( $P = 0.0045$ ; Fig. 3c, left). Thus, vaccination with NYVAC-SIV-*gpe* increases the size of the virus-specific CD8<sup>+</sup> T cells and the CD4<sup>+</sup> helper t cell responses only in animals in which viremia is suppressed by ART.

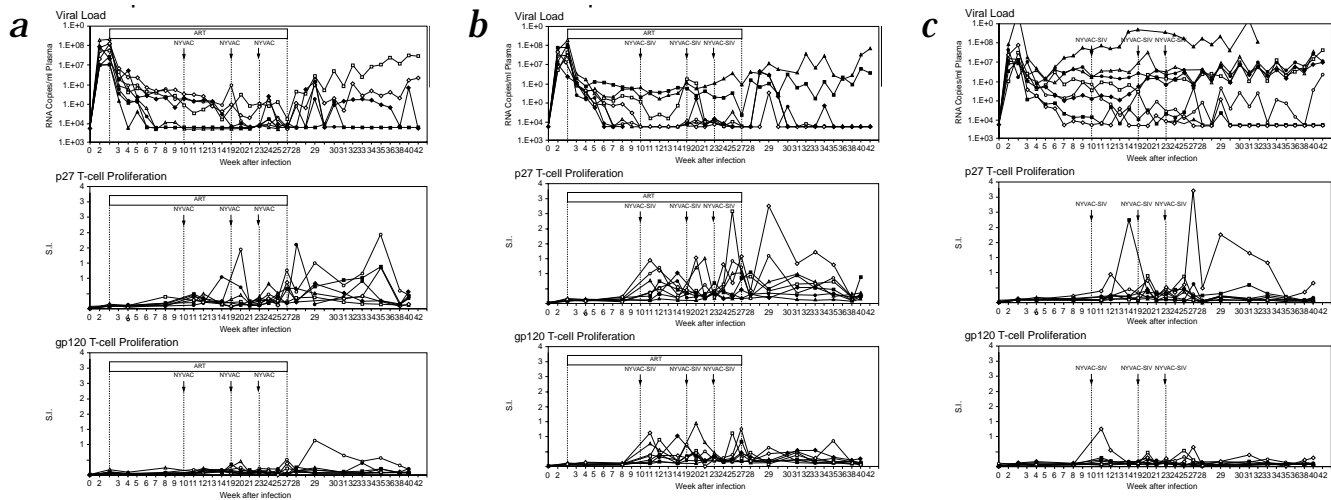
At week 27, ART was discontinued in all animals from groups A and B. In animals of group A, plasma viral rebound occurred in all 7 remaining animals (1 animal, 637, was euthanized at week 25 because of a diabetic coma, presumably due to Didanosine (DDI) toxicity) and peaked between  $10^4$  and  $10^6$  viral RNA copies/ml within the first 3 weeks of ART suspension. Among the animals in group A, however, animals 641, 642 and 652 maintained high plasma viral load thereafter (Fig. 4a, top), whereas the remaining 4 animals in group A suppressed viral replication and maintained viremia below the threshold of detection of our assay ( $5 \times 10^3$  viral RNA copies per ml of plasma).

Of the eight animals in group B, two (647 and 655) failed to respond to ART, experienced a further increase of viremia following ART discontinuation and maintained high virus load (Fig. 4b, top). Of the remaining 6 animals, 5 experienced plasma re-



**Fig. 3** Induction of CD8<sup>+</sup> t cell response by NYVAC-SIV-*gpe* immunization. **a**, Graphical representation of the expansion of *ex vivo* virus-specific CD8<sup>+</sup> Gag 181 t cells after the second and third immunizations. The data were obtained from all the Mamu-A\*01 animals included in each group. The means of the percentages are plotted and the repeated measures ANOVA of the arcsine-transformed percentages revealed highly significant differences in the number of virus-specific CD8<sup>+</sup> T cells induced by vaccination in groups B (●) versus C (○) ( $P = 0.00005$ ). No significant difference was observed between groups A (■), and B (●), and C (○). **b**, Left, the percentage of Mamu-A\*01-p11C, C→M-tetramer-staining cells in freshly prepared PBMC from Mamu-A\*01-positive animals in groups A, B, and C (top to bottom). Data obtained during the first 4 weeks postinfection are presented as percentages of tetramer-staining cells of total CD8<sup>+</sup> αβ lymphocytes. Data obtained after second and third immunizations are expressed as percentages of tetramer-staining cells of total CD3<sup>+</sup> CD8<sup>+</sup> lymphocyte population. Panels on right represent results of bulk-

killing CTL assay at the time of third immunization (week 23). [Fresh PBMC were kept overnight in presence of IL-2 and then incubated with radiolabeled autologous transformed B-cells pulsed overnight with Gag 181 peptide.] The numbers on the x axis represent the effector-to-target cell ratio. The killing of B cells in the absence of peptide or in the presence of an unrelated peptide ranged between 5 and 15%. The background killing is subtracted from the values of specific killing. **c**, Left, the percentage of Mamu-A\*01-Gag 181-tetramer-staining PBMC following *in vitro* culture for 7 days with Gag 181 peptide. Week 0 to 4, percentage of tetramer-staining cells in CD8<sup>+</sup> cell population; weeks 19 to 27, percentage of tetramer-staining cells in CD3<sup>+</sup> CD8<sup>+</sup> t cell population. Right, bulk-killing CTL assay using the Gag 181-peptide-stimulated PBMC obtained at the time (week 19) and 1 week after the second immunization (week 20). Statistical analysis of the data points at weeks 19 through 27, using the repeated measures ANOVA, indicated significant differences between groups B and C ( $P = 0.0045$ ).



**Fig. 4** Viral load, p27 Gag and gp120 LPR in all animal groups before and during immunization and ART and after ART suspension. Top panels of **a-c**, depict the viral RNA copies/ml of plasma. The time of SIV<sub>251</sub> inoculation corresponds to time 0. The arrow indicates the time of each immunization. Middle and lower panels indicate the value of LPR to p27 Gag and gp120,

respectively, measured at the time indicated. The numbers on the y axis correspond to SIs: **a**: 637 ( $\Delta$ ), 640 ( $\circ$ ), 641 ( $\square$ ), 642 ( $\diamond$ ), 645 ( $\blacktriangle$ ), 648 ( $\bullet$ ), 649 ( $\blacksquare$ ), 652 ( $\blacklozenge$ ). **b**: 635 ( $\Delta$ ), 636 ( $\circ$ ), 639 ( $\square$ ), 644 ( $\diamond$ ), 647 ( $\blacktriangle$ ), 650 ( $\bullet$ ), 655 ( $\blacksquare$ ), 656 ( $\blacklozenge$ ). **c**: 473 ( $\Delta$ ), 478 ( $\circ$ ), 479 ( $\square$ ), 480 ( $\diamond$ ), 638 ( $\blacktriangle$ ), 643 ( $\bullet$ ), 646 ( $\blacksquare$ ), 653 ( $\blacklozenge$ ).

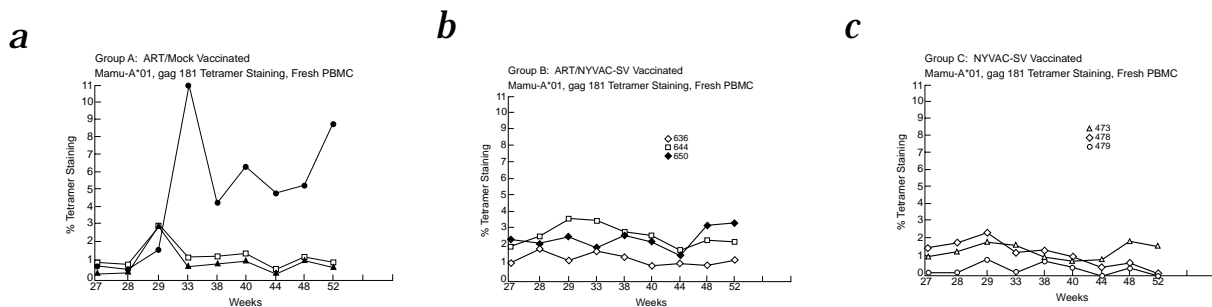
bound above the threshold in the first weeks and rapidly contained viremia thereafter, whereas viral rebound was not observed in animal 636 (Fig. 4b, top).

The overall CD4<sup>+</sup> helper response to p27 Gag and gp120 before and after ART suspension is shown (Fig. 4a-c). Vigorous p27 Gag LPRs were measured in the four animals in group A and in the six animals from group B that contained viremia (Fig. 4a, b, middle). CD4<sup>+</sup> helper responses to gp120 Env were detected only sporadically in some animals (Fig. 4a, b, bottom) after ART suspension. The level of LPR to p27 Gag was significantly higher in macaques that suppressed viremia (4 from group A and 6 from group B), than in those that did not (3 in group A and 2 in group B) before (weeks 10–27;  $P = 0.00004$ ) and after (weeks 28–44;  $P = 0.0029$ ) ART discontinuation, suggesting a possible correlation between Gag-specific proliferation and viremia containment, as also observed in studies of individuals infected with HIV-1 (refs. 14,29,30). This notion was further supported by the finding that animals 480 and 646 from group C, which restricted viral replication early in infection in the absence of ART, also developed LPR to p27 Gag, and one of them developed LPR to gp120 during the time of treatment (Fig. 4c).

The kinetics of expansion of the Gag 181-tetramer CD8<sup>+</sup> T cells

following ART suspension was studied in all Mamu-A\*01 animals. The largest expansion, up to tenfold, of the Gag 181-tetramer-staining CD3<sup>+</sup> CD8<sup>+</sup> t cell population was observed in the blood of animal 649, which restricted viral replication when ART was discontinued. This large expansion was not observed in animals 636, 644 and 650 from group B, which also restricted viral replication (Fig. 5a, c). Therefore, the contribution of this monospecific response to the containment of viral replication is dubious because consistent patterns of expansion were not found in animals that had undetectable viremia and animals that maintained high virus load (Fig. 5a, c).

Of the four animals in group A and the six animals in group B that restricted viral replication, all maintained undetectable levels of plasma-virus load within eight months from ART suspension and had no significant laboratory or clinical sign of disease progression. Among the remaining animals in group A, 641 succumbed to disease 10 months after infection, whereas animals 642 and 652 experienced a progressive loss of CD4<sup>+</sup> cells. Of the remaining animals from group B, animals 647 and 655, which responded poorly to therapy, also experienced a progressive loss of CD4<sup>+</sup> T cells. Of the animals in group C, four (473, 479, 638 and 643) died, indicating that NYVAC-SIV-*gpe* vaccination in the



**Fig. 5** Mamu-A\*01-Gag 181-tetramer staining in *ex vivo* PBMC of macaques following ART discontinuation. The percentage of CD3<sup>+</sup> CD8<sup>+</sup> T cells binding the Mamu-A\*01-Gag 181 tetramer is presented on the y axis versus the time following ART suspension in the 3 Mamu-A\*01 animals from

each group (x axis). **a**, Group A, animal 642 ( $\square$ ) did not control viremia following ART suspension, whereas animals 645 ( $\blacktriangle$ ) and 649 ( $\bullet$ ) did (see Fig. 4). **b**, Group B, all these animals restricted viral replication (see Fig. 4). **c**, Group C, these animals were not treated with ART.

absence of ART was of no obvious clinical benefit to these SIV<sub>251</sub>-infected macaques. In conclusion, ART treatment during primary SIV<sub>251</sub> infection has modified the natural course of disease in animals from groups A and B.

### Discussion

Our data provide the first evidence that a highly attenuated, live recombinant poxvirus vector vaccine is able to induce and/or boost both CD4<sup>+</sup> T-helper and CD8<sup>+</sup> CTL immune responses in the context of a pharmacologically controlled lentiviral infection. This observation is in part unexpected, because it is commonly believed that infection with a given pathogen *per se* may provide the most effective response. SIV, and by extension HIV, infection differ in this regard, as both pathogens infect cells of the immune system and have developed effective strategies to interfere with the host immune response<sup>31–33</sup>. We have demonstrated that the induction of significant CD4<sup>+</sup> T-helper and CD8<sup>+</sup> t cell responses by the NYVAC-SIV-*gpe* vaccination were dependent on the level of viral replication. It is possible that in animals with high plasma viremia, the pool of virus-specific memory CD4<sup>+</sup> T cells is either decreased, exhausted<sup>14</sup> or already activated *in vivo*<sup>14</sup>. In fact, a low number of CD4<sup>+</sup> T lymphocytes responding to short-term *in vitro* stimulation with p27 Gag, as measured by intracellular expression of TNF- $\alpha$ , was detected in the blood of some animals with high virus load, which scored negative in lymphoproliferative assay (data not shown). A similar finding has also been recently demonstrated in humans infected with HIV (ref. 34). An alternative interpretation may be that PBMC of viremic animals harbor virus *in vitro* that could interfere with the measurement of proliferative responses. We do not favor this interpretation, however, because of the short-term nature of our assay and because in the same animals NYVAC-SIV-*gpe* vaccination also failed to expand the Gag-specific CD8<sup>+</sup> T cells, which are not SIV<sub>251</sub> targets. Moreover, a discrepancy was also observed in the ability of CD8<sup>+</sup> T cells from animals in group C to proliferate *in vitro*. In fact, although a relatively high percentage of circulating Gag-specific CD3<sup>+</sup> CD8<sup>+</sup> T cells was found in the blood of these animals, these cells could not be efficiently expanded *in vitro* (Fig. 3b, c), suggesting their limited potential for proliferation.

We have demonstrated that the live, attenuated NYVAC-SIV-*gpe* poxvirus vaccine candidate represents a potent inducer/booster of CD3<sup>+</sup> CD8<sup>+</sup> T cells, as we detected up to 14% Mamu-A\*01-Gag 181-tetramer-staining CD8<sup>+</sup> T cells in the blood of vaccinated animals. We used the Gag 181-tetramer-staining assay as a tool to monitor the CD8<sup>+</sup>-specific immune response to the NYVAC-SIV-*gpe* vaccine. The lack of an obvious correlation with the induction of this response and the ability of the animals to control viremia following therapy suspension suggests that this monospecific Gag-epitope response may not be protective<sup>20,35</sup>. A broader response, which may include other dominant as well as subdominant epitopes in Gag, Pol, Env, Vif, Rev and Tat<sup>25,36</sup> not measured in this study, may be a better correlate of protection, as demonstrated in HIV-I-infected individuals following ART discontinuation<sup>6</sup>. Thus, the availability of other Mamu-A\*01-restricted epitopes, as well as other genetically characterized macaques and the knowledge of the epitopes recognized by their immune systems, might further assist in defining correlates of protection.

We found that all vaccinated animals that responded effectively to ART as well as four of seven mock-vaccinated macaques controlled viremia after ART suspension. The restriction of viral replication in some of the animals treated with antiretroviral

therapy alone was unexpected, as in a previous study only treatment within the first 24 hours from exposure protected macaques from viremia<sup>37</sup>. It is possible, however, that the combination of blunting of acute viremia and long-term ART treatment may have limited the immunological damage in some animals and allowed for an appropriate immunological response to the virus.

Only a few HIV-I-infected individuals have been able to suppress viremia following ART suspension<sup>3–6</sup>. Recent work suggests that HAART treatment during primary HIV-I infection may indeed limit dissemination of the virus, preserve virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> t cell response, and limit immunological damage<sup>6,13</sup>. Moreover, in one of these studies, 4 HIV-I-infected individuals treated within the first 20 days from infection temporarily contained viremia when HAART was discontinued<sup>6</sup>. Thus, the fact that in our study four of seven macaques treated with ART during primary SIV<sub>251</sub> infection (group A) also restricted viral replication in the absence of ART mirrors the observation in humans, validates this animal model, and underscores the importance of early diagnosis and treatment of HIV-I infection in humans.

The effect of ART alone in our study has interfered with our ability to reach unequivocal conclusions on the contribution of NYVAC-SIV-*gpe* vaccination to the containment of viremia following ART suspension. Nevertheless, the ability of a NYVAC-based candidate vaccine to induce both CD4<sup>+</sup> and CD8<sup>+</sup> t cell response in infected animals warrants studies in HAART-treated HIV-I-infected individuals, as vigorous CD4<sup>+</sup> and CD8<sup>+</sup> t cell responses have been associated with containment of HIV-I infection in humans<sup>6,12–14</sup>.

The NYVAC vector, derived from the Copenhagen strain of vaccinia, has been attenuated by precise deletions in 18 open reading frames encoding functions implicated in poxvirus virulence and host-range phenotype<sup>38</sup>. Safety concerns, however, need to be addressed. In our study, inoculation of the NYVAC or NYVAC-SIV vector in SIV-infected animals did not result in any adverse effects. In addition, in a previous unpublished study, we demonstrated the lack of vaccinia dissemination following intravenous, subcutaneous or intramuscular inoculation of 10<sup>8</sup> pfu of NYVAC-SIV recombinant vector in 10 macaques with immunodeficiency (CD4<sup>+</sup> t cell count ranging from 60 to 130 and virus load ranging between 10<sup>6</sup> and 10<sup>7</sup> copies of viral RNA per ml of plasma). Although the final safety assessment of the NYVAC vector and therefore usefulness awaits further testing, our study indicates that immune therapy in HIV-I-infected individuals is a rational approach. In addition, our data demonstrate that immune-based intervention likely will be more effective in the presence of ART.

NYVAC and other live-vector vaccine candidates<sup>18–22</sup> that induce t cell-mediated immunity, alone or in conjunction with immunomodulatory molecules that restore immune function and improve the immunogenicity of these vaccines<sup>39–41</sup>, may decrease or render intermittent the need for HAART in HIV-I-infected individuals.

### Methods

**Animals.** All animals were colony-bred rhesus macaques (*M. mulatta*) obtained from Covance Research Products (Alice, Texas). The animals were housed and handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care. All animals were in good health, 2–4 y, and weighed 3–6 kg. Before study, all animals were seronegative for SIV, SRV, simian t cell lymphotropic virus type I (STLV-I) and herpesvirus B.

**Inoculation and ART.** Macaques were inoculated intravenously with 10 TCID<sub>50</sub> of pathogenic SIV<sub>251</sub>. At day 15 following inoculation, 16 animals in groups A and B received subcutaneous inoculations of 10 mg/kg/d of PMPA ((R)-9-(2-phosphonylmethoxypropyl)adenine; ref. 37), oral administrations of 1.2 mg/kg/d of Stavudine (d4T) divided into 2 doses daily, and intravenous inoculations of 10 mg/kg/d of DDI. At day 28 after infection and thereafter, the daily dose of PMPA was increased to 20 mg/kg/d and Stavudine to 2.4 mg/kg/d.

**Viral-load measurement.** We quantified SIV<sub>251</sub> RNA in plasma by nucleic acid sequence-based amplification<sup>42</sup>. Briefly, RNA extracted from plasma was subjected to isothermal amplification with primers specific for SIV<sub>251</sub> and quantified by electrochemiluminescence chemistry using a coextracted internal standard. The detection limit of this assay was 5 × 10<sup>3</sup> RNA copies/input volume.

**Lymphocyte proliferation assay.** Antigen-specific proliferation was measured in PBMC from fresh blood samples. PBMC were isolated by density-gradient centrifugation on Ficoll, resuspended in RPMI-1640 medium (Gibco, BRL) containing 5% human A/B serum (Sigma), and cultured at 10<sup>6</sup> cells/well for 3 d in the absence or in the presence of native purified viral p27 Gag or gp120 proteins (ABL, Rockville, Maryland) or Concanavalin A as a positive control. The cells were then pulsed overnight with 1 μCi of [<sup>3</sup>H]thymidine before collection. The relative rate of lymphoproliferation was calculated as fold of thymidine incorporation into cellular DNA of cells stimulated with antigens versus cells maintained in media alone (stimulation index, SI). An SI of more than 3 was considered positive.

**Detection of Gag 181-tetramer-staining CD3<sup>+</sup> CD8<sup>+</sup> T lymphocytes.** We screened rhesus macaques for the presence of the Mamu-A\*01 allele using a PCR-based technique<sup>24</sup>. Freshly prepared PBMC were stained with anti-human CD3 antibodies (CyChrome-labeled, clone SP34, Pharmingen), anti-human CD8αβ antibody (FITC-labeled, Becton-Dickinson) and Mamu-A\*01-Gag 181 conjugate (PE-labeled, J. Altman). We analyzed samples on a FACScan (Becton-Dickinson) and the data are presented as percentage of tetramer-positive cells of all CD3<sup>+</sup> CD8<sup>+</sup> cells, unless specified otherwise. To expand the Gag 181-specific CD8<sup>+</sup> t cell population, 5 × 10<sup>6</sup> cells at 3 × 10<sup>6</sup> cells/ml were incubated with Gag 181 peptide at a final concentration of 10 μg/ml for 3 d. Recombinant IL-2 (Boehringer) was added at 20 IU/ml and the cells were cultured for an additional 4 d and stained as described for fresh PBMC.

**CTL assay.** We cultured macaques PBMC overnight in the presence of 100 IU/ml IL-2 and then incubated at different effectors to target cell ratios for 6 hours with <sup>51</sup>Cr-labeled autologous transformed B cells pulsed overnight with 1 μg/ml Gag 181. The background killing in the absence of peptide was between 5 and 15% and is subtracted from the values of specific killing. The killing of cells pulsed with unrelated peptide as control was generally similar to the killing observed in the absence of peptide.

**Statistical analysis.** Comparisons of viral load after initiating ART were made by applying the Wilcoxon rank sum test to the means of the logarithmically transformed RNA copy numbers for each animal. All tests of p27 Gag and gp120 Env levels were performed using repeated measures analysis of variance (ANOVA) on the log-transformed SI. This procedure combines the *t*-tests at each time point while making the necessary correction for the correlations among the multiple values from each animal. The tetramer-positive percentages of CD3<sup>+</sup> CD8<sup>+</sup> lymphocytes were analyzed using repeated measures ANOVA after the arcsine transformation of the square root of each percentage. This transformation is commonly used with percentage data to bring closer to equality the unequal variances of a range of percentages. ANOVA *P* values are based on the assumption of a normal distribution of the deviations between observed and expected values, and agreement with this assumption was verified. All *P* values reported are two-tailed. The SAS (version 8, SAS Institute, Cary, North Carolina) and StatXact (version 4.0.1, Cytel Software, Cambridge, Massachusetts) statistical software packages were used for these analyses.

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2	13430 2872	Romeo and Juliet /	11 SEP 00	*	SGC
3	12002 1502	The tragedy of Romeo and Juliet /	11 SEP 00	*	SGC
4	11351 4232	Hiroshima mon amour.	19 SEP 00	*	MML
5	09191 2481	The story of Adele H.	19 SEP 00	*	MML
6	07635 6159	Behavioural ecology :	19 SEP 00	*	SIBL
7	04267 9569	Animal minds /	19 SEP 00	*	SIBL
8	07635 6522	The psychology and behaviour of	19 SEP 00	*	SIBL
9	12870 4075	If a lion could talk :	19 SEP 00	*	SIBL
10	11081 1946	The Fugitive kind	12 SEP 00	*	MML
11	01383 2924	German expressionist drama :	02 OCT 00	*	MML
12	01443 1429	German expressionist drama /	02 OCT 00	*	MML
13	02087 0339	Seascape;	02 OCT 00	*	MML
14	14090 0230	The boy with the Arab strap	16 SEP 00	*	EP
15	10823 4770	The Young Bechet	16 SEP 00	*	EP
16	11272 9633	Stellar regions	16 SEP 00	*	EP
17	12190 7105	Premieres chansons	16 SEP 00	*	EP
18	11272 9609	Duke Ellington & John Coltrane	16 SEP 00	*	EP
19	10416 6232	Hamlet /	16 SEP 00	*	EP
20	10552 3852	Nightmares of nature /	16 SEP 00	*	EP
21	07573 2855	The Biology of sea snakes /	30 SEP 00	*	SGC
22	11351 7367	Three by Tennessee :	30 SEP 00	*	SGC
23	07805 0552	Heroines :	30 SEP 00	*	SGC
24	02435 1187	The serpent's egg,	30 SEP 00	*	SGC
25	01980 3895	A man and a woman;	30 SEP 00	*	SGC
26	07971 7589	The theatre of Tennessee Williams.	30 SEP 00	*	SGC
27	05719 4686	The theatre of Tennessee Williams.	30 SEP 00	*	SGC