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# Containment of Simian Immunodeficiency Virus Infection in Vaccinated Macaques: Correlation with the Magnitude of Virus-Specific Pre- and Postchallenge CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Responses<sup>1</sup>

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Macaques infected with the SIV strain SIVmac251 develop a disease closely resembling human AIDS characterized by high viremia, progressive loss of CD4<sup>+</sup> T cells, occurrence of opportunistic infection, cachexia, and lymphomas. We report in this study that vaccination with the genetically attenuated poxvirus vector expressing the structural Ags of SIVmac (NYVAC-SIV-*gag*, *pol*, *env*) in combination with priming with DNA-SIV-*gag*, *env* resulted in significant suppression of viremia within 2 mo after mucosal exposure to the highly pathogenic SIVmac251 in the majority of vaccinated macaques. The control of viremia in these macaques was long lasting and inversely correlated to the level of both pre- and postchallenge Gag-specific lymphoproliferative responses, as well as to the level of total SIV-specific CD4<sup>+</sup> T lymphocyte responses at the peak of acute viremia as detected by intracellular cytokine-staining assay. Viremia containment also correlated with the frequency of the immunodominant Gag<sub>181-189</sub>CM9 epitope-specific CD8<sup>+</sup> T cells present before the challenge or expanded during acute infection. These data indicate, for the first time, the importance of vaccine-induced CD4<sup>+</sup> Th cell responses as an immune correlate of viremia containment. The results presented in this work also further demonstrate the potential of a DNA-prime/attenuated poxvirus-boost vaccine regimen in an animal model that well mirrors human AIDS. *The Journal of Immunology*, 2002, 169: 4778–4787.

Recent HIV-1 vaccine efforts have demonstrated the potential for using DNA-prime/live attenuated recombinant virus-boost vaccine strategy (1–4) or the coadministration of Ag with rIL-2 (5) in simian HIV (SHIV)<sup>3</sup> 89.6P/rhesus macaques model. However, relevance of this macaque model for human AIDS has been questioned because of the rapidity of CD4<sup>+</sup> T cell depletion that occurs in SHIV-89.6P-infected animals (1, 6, 7). Prevention or containment of viral replication in SIV macaque models that better mimic HIV-1 infection in humans, such as SIVsmE660 (8, 9) or SIVmac251 (10–12), has proven to be quite difficult. In fact, only vaccination with a live attenuated SIV has conferred protection from infection (13). Neutralizing Abs administered before viral challenge do not protect macaques from SIVsmE660 isolate (14), but were shown to protect macaques from SHIV challenges (15, 16).

The definition of immune correlates of protection against HIV-1 infection has been elusive. Although some studies in nonhuman

primates have indicated a correlation between the protection and the level of vaccine-induced CTL responses to the immunodominant Gag<sub>181-189</sub>CM9 (Gag\_CM9) epitope (5, 9, 17) or to the Nef protein (11), the role of vaccine-induced virus-specific CD4<sup>+</sup> Th lymphocyte response has not been thoroughly investigated. In HIV-1-infected individuals, the presence of the virus-specific lymphoproliferative response (LPR) has been shown to be associated with delayed disease progression (18, 19). In rhesus macaques, the extent of virus-specific LPR induced by therapeutic vaccination of SIVmac-infected antiretroviral therapy-treated animals correlated with the containment of viremia after drug withdrawal (20, 54). Moreover, adoptive transfer of naive autologous CD4<sup>+</sup> T cells in SIVmac-infected macaques resulted in an increase of virus-specific CTL responses and long-term viremia containment, suggesting a defect in the Th cell compartment rather than the effector cell compartment (21).

Immunization with the highly attenuated poxvirus-based vector NYVAC-SIV-*gag*, *pol*, *env* (NYVAC-SIV-*gpe*) vaccine candidate has been shown to induce and/or expand SIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in both naive and SIV-infected rhesus macaques (10, 20, 22, 24, 54). Previously, we demonstrated that priming with DNA-*gag*, *env* expressing the Gag and Env proteins of SIV (DNA) significantly enhanced the ability of the NYVAC-SIV-*gpe* to induce and expand both the LPR and CTL responses (23). In this study, we report that immunization with NYVAC-SIV-*gpe* alone and, especially, the immunization consisting of a DNA-prime/NYVAC-SIV-*gpe* boost induced significant secondary immune responses and was associated with the containment of viremia following a mucosal exposure to a highly pathogenic SIVmac251 viral challenge. Importantly, the containment of viremia correlated with the level of virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses induced by vaccination, as well as with the level of Th and CTL responses occurring during the acute phase

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<sup>3</sup> Abbreviations used in this paper: SHIV, simian HIV; ICC, intracellular cytokine; LPR, lymphoproliferative response; NYVAC-SIV-*gpe*, NYVAC-SIV-*gag*, *pol*, *env*; Gag\_CM9, Gag<sub>181-189</sub>CM9.

of infection, indicating the importance of both arms of cellular immunity in the suppression of viral replication.

## Materials and Methods

### *Animals, immunizations, and challenge*

All animals were colony-bred rhesus macaques (*Macaca mulatta*) obtained from Covance Research Products (Alice, TX). The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International. All rhesus macaques were seronegative for simian retrovirus, simian T cell leukemia virus type 1, and herpesvirus B before the study. The macaques were screened by PCR and sequencing for the presence of the Mamu-A\*01 allele (23). The Mamu-A\*01 status of animal 16 M was unknown until the end of the study.

The animals were immunized as described (23). Briefly, the animals were immunized i.m. with  $10^8$  PFU of mock NYVAC (group A) or NYVAC-SIV-*gpe* (group B) vaccine at 0, 4, 24, and 52 wk. Animals in group C were first immunized by simultaneous i.m. and intradermal inoculations of DNA-SIV-*gag*, *env* at 0, 4, and 12 wk, followed by two boosts with  $10^8$  PFU of NYVAC-SIV-*gpe* given i.m. at 24 and 52 wk. For DNA immunization, 4 mg of each plasmid (CMV/kan-SIV-*env* and CMV/kan-SIV-*gag*) was administered. Four doses of 0.75 mg of each plasmid were injected i.m. into two sites on each leg; five doses of 0.2 mg of each plasmid were injected intradermally at five different sites in the abdominal area. Construction of Rev-independent SIV *gag* and *env* expression vectors optimized for high level expression was described previously (23).

The challenge virus stock was prepared from PHA-activated PBMC obtained from Mamu-A\*01-positive macaque 561L previously inoculated vaginally with SIVmac251 (24). The monkeys were challenged by an intrarectal exposure to 30 mucosal infectious doses of SIVmac251 challenge stock. SIVmac251 RNA in plasma was quantitated by nucleic acid sequence-based amplification (25). Because the threshold of detection of this assay is  $2 \times 10^3$  RNA copies and typically 100  $\mu$ l of plasma is assayed, the data are presented without further extrapolation as viral RNA copies/100  $\mu$ l of plasma.

### *Lymphocyte proliferation assay*

The Ficoll-purified PBMC were resuspended in RPMI 1640 medium (Life Technologies, BRL, Gaithersburg, MD) containing 5% inactivated human A/B serum and antibiotics (Sigma-Aldrich, St. Louis, MO), and cultured at  $10^5$  cells/well in triplicate for 3 days in an absence or presence of native HPLC-purified SIVmac Gag p27 or Env gp120 proteins (Advanced Bio-Science Laboratories, Rockville, MD) or Con A as a positive control. The cells were then pulsed overnight with 1  $\mu$ Ci of [ $^3$ H]thymidine before harvest. The relative rate of lymphoproliferation was calculated as fold of thymidine incorporation into cellular DNA over medium control (stimulation index) (4).

### *Detection of epitope-specific CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes by tetramer staining*

Fresh PBMC were stained with anti-human CD3 Ab (PerCP labeled, clone SP34; BD PharMingen, San Diego, CA), anti-human CD8 $\alpha$  Ab (FITC labeled; BD Biosciences, San Jose, CA), and Mamu-A\*01 tetrameric complexes refolded in the presence of a specific peptide and conjugated to PE-labeled streptavidin (Molecular Probes, Eugene, OR). Gag<sub>181–189</sub> CM9 (CTPYDINQM) (Gag\_CM9)- and Tat<sub>28–35</sub>SL8 (TTPESANL) (Tat\_SL8)-specific tetramers were used. Samples were analyzed on FACSCalibur (BD Biosciences), and the data are presented as percentage of tetramer-positive cells of all CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes.

### *ELISPOT assay*

Monkey IFN- $\gamma$ -specific ELISPOT kits manufactured by U-Cytech (Utrecht, The Netherlands) were used. Ninety-six-well flat-bottom plates were coated with anti-IFN- $\gamma$  mAb MD-1 overnight at 4°C and blocked with 2% BSA in PBS for 1 h at 37°C. A total of  $10^5$  cells/well were loaded in quadruplicate in RPMI 1640 containing 5% human serum and 10  $\mu$ g/ml of a specific peptide pool (15 mers overlapping by 11 aa encompassing the full SIV Gag or Env protein). The plates were incubated overnight at 37°C and 5% CO<sub>2</sub> and developed according to the manufacturer's guidelines. Negative controls included nonspecific peptides and naive animals.

### *Intracellular TNF- $\alpha$ staining*

A total of  $1 \times 10^6$  cells in RPMI 1640 medium (containing 10% human serum and antibiotics) were incubated in an absence or presence of a spe-

cific peptide pool at 2  $\mu$ g/ml of each peptide for 1 h, as described (26). Brefeldin A (Sigma-Aldrich) at a final concentration of 10  $\mu$ g/ml was added, and the cells were incubated for an additional 5 h. The cells were washed, stained for the surface Ags CD3 $\epsilon$  and CD8 $\alpha$ , permeabilized by incubation in FACSPerm solution (BD PharMingen), and stained with CD69 (BD PharMingen) and anti-TNF- $\alpha$  (clone mAb.11; BD PharMingen).

### *Detection of anti-SIVmac251-binding and -neutralizing Abs*

To detect anti-SIVmac251-binding Abs, serial dilutions of plasma were incubated with the lysate of SIVmac251 spiked with native purified gp120 Env protein of SIVmac251 bound to microtiter ELISA plates, as described elsewhere (27). End-point titers were defined as the reciprocal of the highest sera dilution that gave an optical absorbency at 450 nm at least 2 SDs greater than average values obtained with negative control sera.

Neutralizing Abs against the assay stocks of lab-adapted SIVmac251 (produced in H9 cells) and primary SIVmac251/561L (produced in human PBMC) were detected, as described elsewhere (10). Neutralizing Ab titers were defined as the reciprocal plasma dilution at which 50% of the target cells were protected from virus-induced killing, as detected by neutral red uptake. CEMx174 cells were used as targets for lab-adapted SIVmac251 virus; CEMx174-R5 were used as targets for SIVmac251/561L virus.

### *Statistical analysis*

All reported *p* values are two-sided. All correlation coefficients were calculated using the Spearman rank test with 95% confidence interval. Viral loads were compared by the Mann-Whitney rank sum test. Repeated measures (5) ANOVA analysis was performed on transformed data, as described (20). The Number Cruncher Statistical system (Kaysville, UT) and Sigmatat (version 2.0; SPSS, Chicago, IL) statistical software packages were used for the analyses.

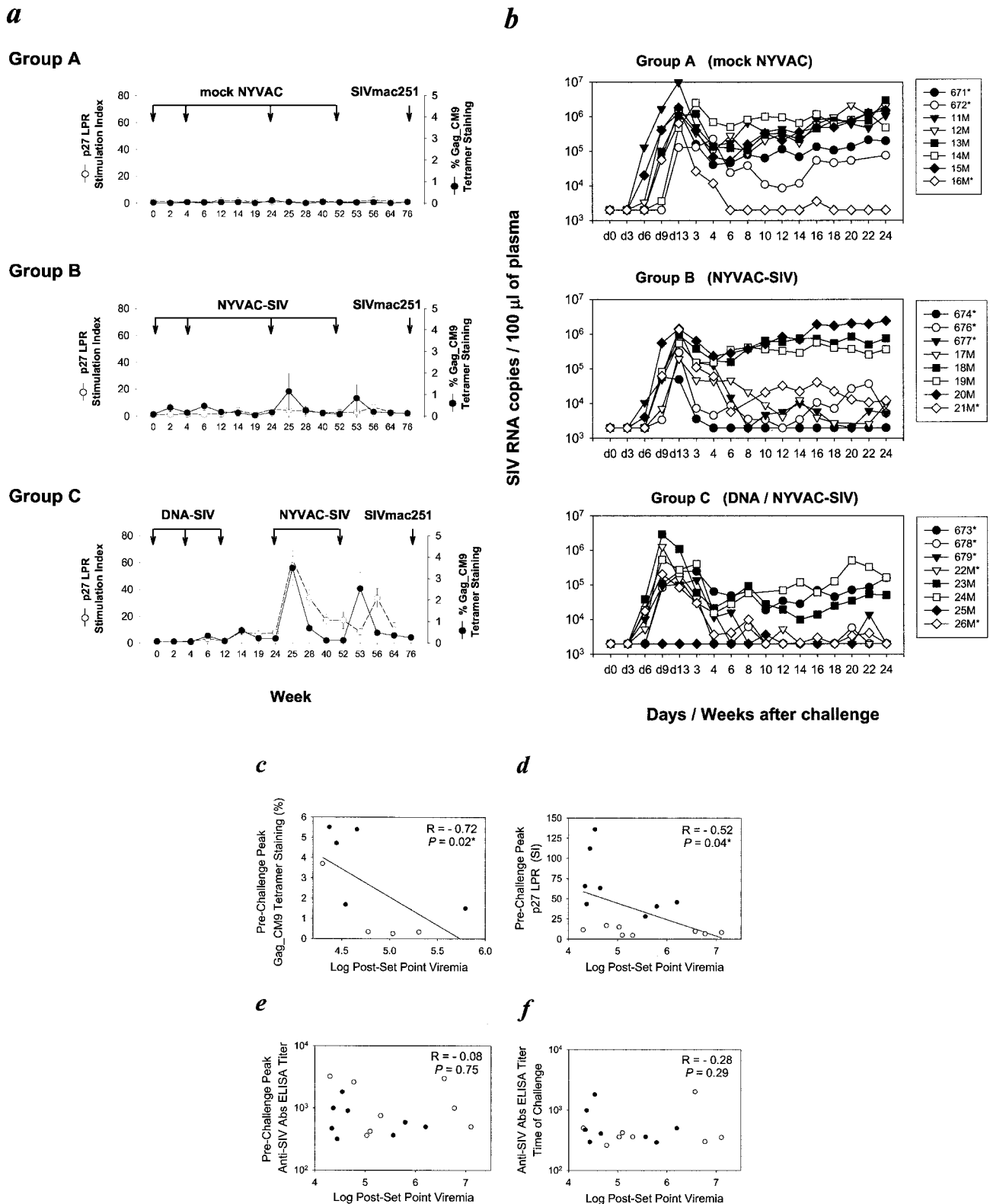
## Results

### *DNA/NYVAC-SIV-*gpe* prime-boost regimen induced high level lymphoproliferative and CTL responses*

In this study, we wished to extend upon the previous observation that immunization with NYVAC-SIV-*gpe* was associated with reduced viremia following intrarectal challenge of rhesus macaques with the SIVmac251 (32H) (10). To do so, we have chosen the DNA-prime/NYVAC-SIV-*gpe*-boost strategy. The first two groups of eight rhesus macaques each received four i.m. inoculations of mock NYVAC (group A) or NYVAC-SIV-*gpe* vaccine candidate (group B) (Fig. 1*a*). Animals in the third group (group C) received three i.m. and intradermal inoculations of DNA-SIV-*gag*, *env*, followed by two i.m. inoculations with NYVAC-SIV-*gpe*. The DNA-prime/NYVAC-SIV-*gpe*-boost regimen induced high frequencies of CD8<sup>+</sup> T cells specific for the immunodominant epitope Gag<sub>181–189</sub>CM9 (Gag\_CM9), as well as other epitopes, and, notably,  $\sim$ 10-fold higher levels of LPR to SIV Gag p27 protein compared with the immunization with NYVAC-SIV-*gpe* alone (Fig. 1*a*) (23).

### *DNA/NYVAC-SIV-*gpe*-immunized macaques exhibited reduced levels of viremia during both the acute and chronic phases of infection*

Six months after the last immunization, the macaques were exposed intrarectally to 30 mucosal infectious doses of SIVmac251 (561) (24). By 13 days after challenge, all macaques, except one (25 M), became viremic (Fig. 1*b*). However, plasma viral RNA levels during acute infection were significantly higher in macaques from the control group than in macaques from both vaccinated groups (*p* = 0.04 for group A vs B, and *p* = 0.003 for A vs C, mean viremia in the period from day 13 to 28, analyzed by two-sided Mann-Whitney rank sum test). At viremia set point at 2–3 mo from exposure, five of eight macaques in the NYVAC-SIV-*gpe*-vaccinated group B and only two of eight macaques in the control group had viremia level below  $5 \times 10^4$  viral RNA copies/100  $\mu$ l of plasma. Importantly, following the set point of infection, five of eight monkeys in the DNA/NYVAC-SIV-*gpe*-vaccinated



**FIGURE 1.** Containment of SIV<sub>mac251</sub> infection correlates with vaccine-induced Gag-specific LPRs and CTL immune responses. *a*, Mean LPR to p27 Gag Ag (○) and mean percentages of Gag<sub>CM9</sub> tetramer-staining cells of total CD3<sup>+</sup>CD8<sup>+</sup> lymphocyte population (●) in mock-vaccinated macaques (group A), macaques vaccinated with NYVAC-SIV-*gpe* (group B), or a combination of DNA-SIV-*gag*, *env* (DNA) and NYVAC-SIV-*gpe* (group C). Group mean values are shown with SEs indicated by bars. Arrows indicate the time of immunization with either mock NYVAC, NYVAC-SIV-*gpe*, or DNA. *b*, Plasma viral levels (copies of viral RNA/100 μl of plasma) in rhesus macaques following the challenge exposure to SIV<sub>mac251</sub>. Mamu-A\*01-positive animals are designated by asterisks in the legend. *c* and *d*, Inverse correlation between the peak prechallenge frequency of Gag<sub>CM9</sub> tetramer-staining cells (*c*) or peak prechallenge LPR to Gag p27 Ag (*d*) and the average level of post-set point viremia (6–24 wk postchallenge). ○, Group B animals; ●, group C animals. *e* and *f*, Lack of correlation between the peak prechallenge (*e*) or time-of-challenge (*f*) levels of anti-SIV<sub>mac251</sub>-binding Abs determined by ELISA and the average level of post-set point viremia (6–24 wk postchallenge). The correlation coefficients (R) and the *p* values determined by the Spearman rank order correlation test are shown in the upper right corners. ○, Group B animals; ●, group C animals.

group C contained viremia close to or below the level of detection (Fig. 1*b*). Animal 25 M had detectable plasma viremia at a single time point (10 wk). The average level of viremia following the set point of infection (6–24 wk) was significantly lower in animals immunized with DNA/NYVAC-SIV-*gpe* than in control animals (mean value  $3.4 \times 10^4$  vs  $4.5 \times 10^5$  RNA copies/100  $\mu$ l of plasma,  $p = 0.038$ ), while the difference between animals immunized with NYVAC-SIV-*gpe* alone and controls did not reach statistical significance.

We have previously observed that rhesus macaques carrying the MHC I Mamu-A\*01 molecule as a group restrict the replication of SIVmac251 (561) better than most Mamu-A\*01-negative animals (24). Because different numbers of Mamu-A\*01-positive macaques were unknowingly included in the three experimental groups (Fig. 1*b*), the effect of vaccination on viremia containment was assessed separately in Mamu-A\*01-positive and -negative vaccinated and control animals. Prior vaccination of Mamu-A\*01-positive macaques resulted in lower median viremia during primary infection (days 13–28 postchallenge) in vaccinated ( $8.4 \times 10^5$  RNA copies/ml plasma) than in control ( $2.2 \times 10^6$  RNA copies/ml plasma) macaques, but this difference did not reach statistical significance. Similarly, at set point and thereafter (6–24 wk), median viremia was lower in vaccinated ( $2.6 \times 10^4$  RNA copies/ml plasma) than in control ( $1 \times 10^5$  RNA copies/ml plasma) macaques, but again this difference only approached statistical significance.

In contrast, a significant difference in viremia levels was observed among the Mamu-A\*01-negative macaques in the control group A and the vaccinated macaques in group C during both acute infection ( $p = 0.04$ , days 13–28 postchallenge) and following the set point ( $p = 0.036$ , 6–24 wk). Thus, a significant effect of vaccination with DNA/NYVAC-SIV-*gpe* on viremia containment was observed in the Mamu-A\*01-negative macaques. All together, the data confirm our previous finding that the evaluation of relative vaccine efficacy in macaques challenged with this SIVmac251 strain needs to account for the Mamu-A\*01-positive status of macaques (24).

In summary, although at set point viremia and thereafter the difference in viremia between group B and C macaques only approached statistical significance, the advantage of prior DNA immunization was reflected by the finding of a significant difference in macaques from group C vs control macaques, even when the data were analyzed accounting for the Mamu-A\*01 status (24).

#### Correlation between the prechallenge immune responses and control of viremia

Because we had observed a clearer effect on postchallenge viremia in the Mamu-A\*01-negative than Mamu-A\*01-positive macaques, at first we assessed whether the immunological responses induced by vaccination differed in macaques expressing this MHC I molecule. Analysis of p27 Gag LPRs (weekly measurements from 25 to 68 wk), mean number of virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing TNF- $\alpha$  at any given point, and neutralizing Ab titers did not differ significantly between Mamu-A\*01-positive and -negative macaques vaccinated with the two regimens. Therefore, the analysis of the correlation of the vaccine-induced immune responses and viremia containment after challenge at set point and thereafter included all macaques, except when the Mamu-A\*01 tetramers were used.

The reduction of viral load following the set point of infection (6–24 wk) inversely correlated with the peak as well as the prechallenge frequencies of vaccine-induced CTLs specific for the immunodominant Gag<sub>CM9</sub> epitope (Fig. 1*c*). Interestingly, the peak and the prechallenge LPR to Gag p27 protein also correlated with the control of viremia (Fig. 1*d* and Table I), whereas the correlation with the LPR to Env gp120 Ag did not reach statistical significance.

Immunization of group B and C animals with NYVAC-SIV-*gpe* and DNA/NYVAC-SIV-*gpe* vaccine candidates induced comparable serum titers of SIVmac251-binding Abs detected by ELISA (23). Importantly, no significant negative or positive correlation between the peak prechallenge or time-of-challenge levels of SIVmac251-specific Abs and viremia containment following the set point of infection was observed (Fig. 1, *e* and *f*; Table I).

Table I. Statistical analysis of pre- and postchallenge SIV-specific immune responses

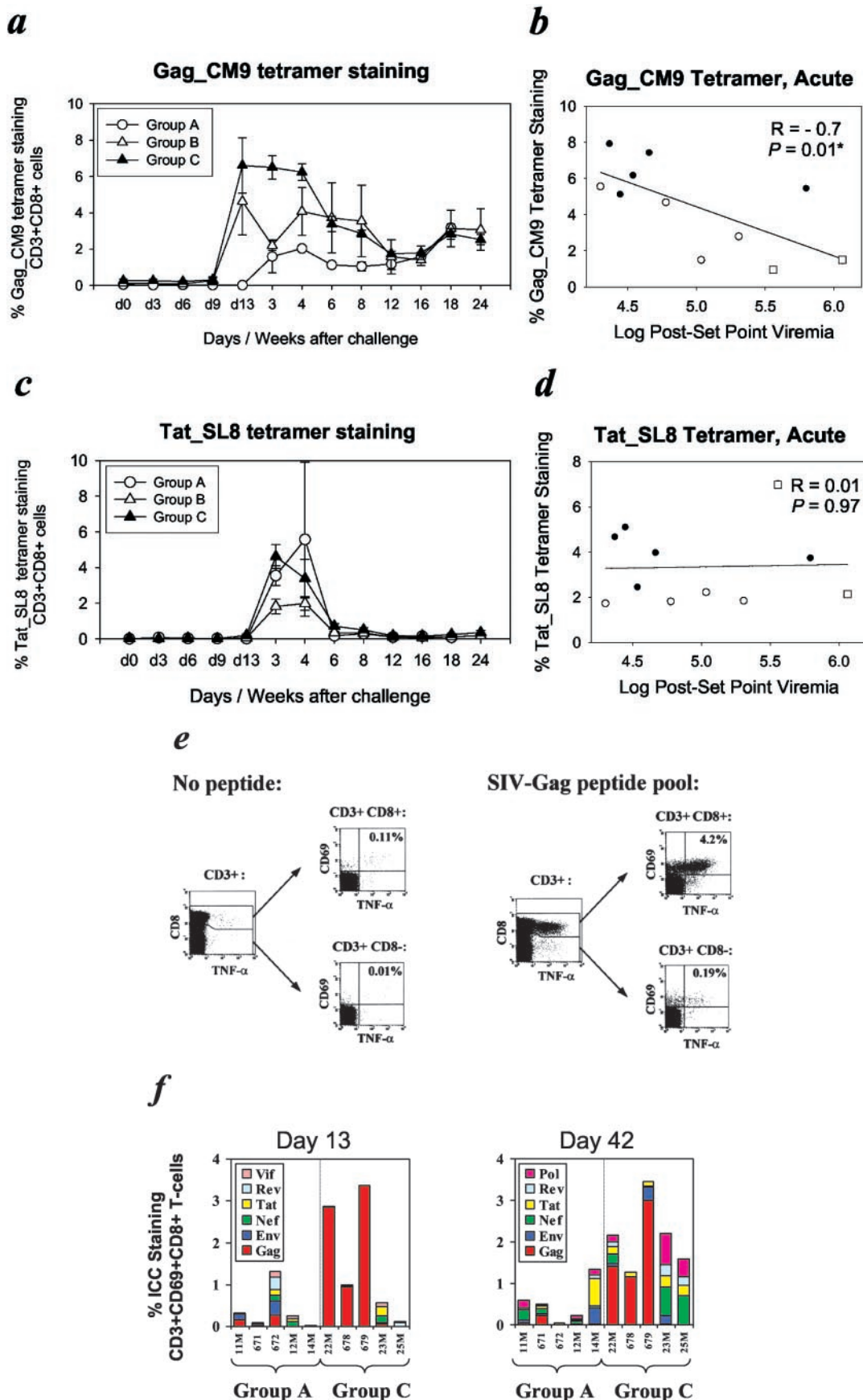
Parameters Compared	Groups	<i>p</i>	<i>R</i>	Correlation	
Correlation of prechallenge immune responses to viremia containment					
Peak % Gag <sub>CM9</sub> Tet day 13–28 postchallenge <sup>a,b</sup>	Post-set point viremia wk 6–24	B and C	0.02*	–0.72	Inverse
Peak p27 LPR prechallenge <sup>c</sup>	Post-set point viremia wk 6–24	B and C	0.04*	–0.52	Inverse
p27 LPR wk 25–68 prechallenge	Post-set point viremia wk 6–24	B and C	0.04*	–0.51	Inverse
Peak gp120 LPR prechallenge	Post-set point viremia wk 6–24	B and C	0.19	–0.34	No
gp120 LPR wk 25–68 prechallenge	Post-set point viremia wk 6–24	B and C	0.46	–0.19	No
Correlation of postchallenge immune responses to viremia containment					
% Gag <sub>CM9</sub> Tet day 13–28 postchallenge	Post-set point viremia wk 6–24	A, B, and C	0.01*	–0.71	Inverse
% Tat <sub>SL8</sub> Tet day 21–28 postchallenge	Post-set point viremia wk 6–24	A, B, and C	0.97	0.01	No
p27 LPR wk 2–20 postchallenge	Post-set point viremia wk 6–24	A, B, and C	<0.001*	–0.78	Inverse
Total CD8 ICC, day 13 postchallenge <sup>d</sup>	Post-set point viremia wk 6–24	A and C	0.1	–0.54	No
Total CD4 ICC, day 13 postchallenge	Post-set point viremia wk 6–24	A and C	<0.001*	–0.82	Inverse
SIV-binding Abs (ELISA), wk 12 postchallenge	Post-set point viremia wk 6–24	A, B, and C	0.02*	0.47	Positive
NAb, lab-adapted SIVmac251, wk 12 postchallenge	Post-set point viremia wk 6–24	A, B, and C	0.03*	0.45	Positive
NAb, SIVmac251/261L, wk 12 postchallenge	Post-set point viremia wk 6–24	A, B, and C	0.15	0.47	No
Correlations between pre- and postchallenge immune responses					
Peak % Gag <sub>CM9</sub> Tet prechallenge	% Gag <sub>CM9</sub> Tet day 13–28 postchallenge	B and C	<0.001*	0.88	Positive
p27 LPR wk 25–68 prechallenge	Peak % Gag <sub>CM9</sub> Tet prechallenge	B and C	0.04*	0.68	Positive
gp120 LPR wk 25–68 prechallenge	SIV-binding Abs (ELISA), time of challenge	B and C	0.38	0.23	No
gp120 LPR wk 25–68 prechallenge	SIV-binding Abs (ELISA), wk 12 postchallenge	B and C	0.25	–0.3	No

<sup>a</sup> Only vaccinated animals were included in the analysis of the relation between the levels of prechallenge immune responses and containment of viremia following the set-point of infection, since the prechallenge immune responses in the control animals were close to or equal to background. Inclusion of control animals into the analysis further improved the statistical significance of all observed correlations; however, the correlations involving gp120 LPR responses still did not reach statistical significance.

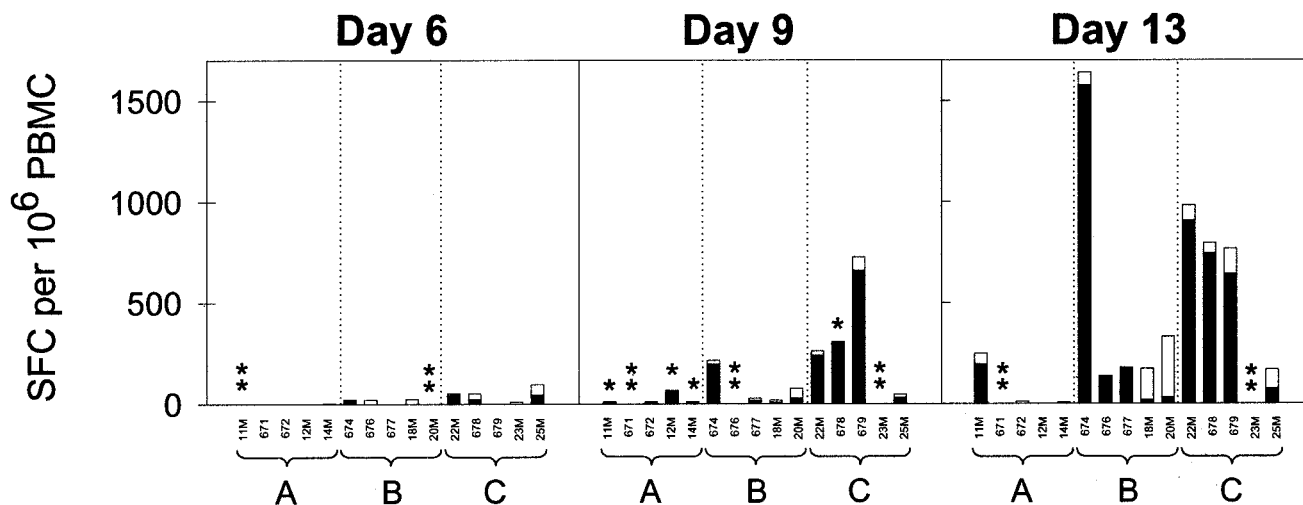
<sup>b</sup> Only Mamu-A\*01-positive animals were included in the correlations involving Gag<sub>CM9</sub> tetramer staining assay.

<sup>c</sup> All animals were included in analysis of LPR and Ab responses. There was no significant difference in the level of these responses between the Mamu-A\*01-positive and -negative animals.

<sup>d</sup> Only five animals from group A and five animals from group C were included in the analysis of ICC responses (see Figs. 2 and 4). Although the ICC responses at day 13 were higher in Mamu-A\*01-positive than Mamu-A\*01-negative animals, the difference did not reach statistical significance.



**FIGURE 2.** Vaccination primes for a secondary CD8<sup>+</sup> T lymphocyte response in the challenged macaques. *a* and *c*, Mean percentages of Gag\_CM9 (*a*)- and Tat\_SL8 (*c*)-specific tetramer-staining cells of total CD3<sup>+</sup>CD8<sup>+</sup> blood lymphocyte population with SEs indicated by bars. *b* and *d*, Correlation between the average frequency of Gag\_CM9 (*b*)- or Tat\_SL8 (*d*) tetramer-staining cells during the acute phase of viremia (days 13–28 or 21–28 postchallenge, respectively) with the average level of post-set point viremia (6–24 wk postchallenge). □, Group A animals; ○, group B animals; ●, group C animals. (figure legend continues)



**FIGURE 3.** Primary and secondary immune response to SIV Gag measured by IFN- $\gamma$  ELISPOT assay in fresh PBMC following a stimulation with overlapping Gag (■) and Env (□) peptide pools. SFC, spot-forming cells; asterisks indicate that measurements with Env (\*) or both Gag and Env (\*\*) peptide pools were not performed.

#### Primary and secondary CD8<sup>+</sup> T lymphocyte responses following mucosal challenge with SIVmac251

The frequencies of Gag\_CM9-specific CTLs in blood detected by tetramer staining were expanded by viral infection in both control and vaccinated Mamu-A\*01-positive animals (Fig. 2*a*). However, the kinetics of appearance of these cells in blood was faster in both vaccinated groups (expansion at day 13) than in control animals (day 21). The overall frequency of Gag\_CM9-specific cells during acute viremia was significantly higher in the DNA/NYVAC-SIV-*gpe*-vaccinated group (up to 12% of total CD3<sup>+</sup>CD8<sup>+</sup> T lymphocyte population) than in the control group (maximum 2.5%) ( $p = 0.0003$ , 2–8 wk; repeated measures ANOVA). Thus, prior vaccination was associated with faster expansion and higher frequencies of Gag\_CM9-specific CTLs following a challenge exposure. Importantly, the average frequencies in blood of these CTLs during the acute phase of viremia inversely correlated with the levels of post-set point viremia (Fig. 2*b*).

We next investigated the primary response to the regulatory Tat protein Tat<sub>28–35</sub> SL8 epitope (Tat\_SL8) (28) (not included in the vaccine). All animals exhibited an expansion of Tat\_SL8-specific cells in the range of 1–7% at 3 wk after challenge (Fig. 2*c*). As expected, the kinetics of appearance of this primary response did not differ in control and vaccinated macaques and was similar to the kinetics of primary Gag\_CM9 response in control animals. The Tat\_SL8 response decreased abruptly by 6 wk postchallenge and remained low during the entire observation period. This, as well as the fact that the extent of Tat\_SL8 CTLs did not correlate with viremia containment (Fig. 2*d*), may reflect the rapid selection of CTL escape mutants at this epitope early during acute infection, as reported (28).

Because the response to immunodominant epitopes may represent only a fraction of the total immune response (29), we investigated immune responses to almost the entire SIV, irrespectively

of the MHC haplotype of the hosts, using the previously developed method of intracellular cytokine (ICC) (6) staining following an in vitro stimulation with SIV-specific peptide pools (26). The expression of TNF- $\alpha$  was assessed simultaneously in the CD3<sup>+</sup>CD69<sup>+</sup>CD8<sup>+</sup> lymphocytes representing the MHC class I-restricted CTL population and in the CD3<sup>+</sup>CD69<sup>+</sup>CD8<sup>-</sup> lymphocytes consisting mainly of the MHC class II-restricted CD4<sup>+</sup> Th lymphocyte population (Fig. 2*e*). The length of the overlapping peptides used (15 mers overlapping by 11 aa) allows for simultaneous detection of both CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses. At day 13 postchallenge, a robust response of CD8<sup>+</sup> T cells to the Gag peptide pool was detected in PBMC of three of five tested animals in group C, while little or no response was detectable in the control macaques (Fig. 2*f*). The three highest-responding group C macaques (22 M, 678, and 679) were Mamu-A\*01 positive, and the peak of the response to the Gag pool corresponded with the peak frequencies of Gag\_CM9 tetramer-staining T cells. Few or no functional responses were detected in animals of group A at days 21 and 42 after infection, despite the fact that 0.7%–2.5% of CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes in the blood of animals 671 and 672 were specific for Gag\_CM9. The inability of these lymphocytes to respond to the specific stimulus by cytokine production may be related to the virus-specific CD8<sup>+</sup> T cell functional impairment observed in viremic macaques (30, 31), as well as HIV-1-infected humans (32–34). At day 42 postchallenge, variable frequencies of CTLs specific to Pol and Env, as well as to the Tat, Nef, and Rev proteins, were detected in most animals, and the total CD8<sup>+</sup> T lymphocyte response was significantly higher in immunized animals of group C compared with the control group ( $p = 0.02$ ). High frequencies of Gag- and Tat-specific CTLs were detected primarily in the Mamu-A\*01-positive animals and coincided with the occurrence of peak frequencies of Gag\_CM9- and Tat\_SL8 tetramer-staining cells, confirming the observation that the response to those

*e*, Depiction of the method used for the quantitation of total CD8<sup>+</sup> and CD8<sup>-</sup> (CD4<sup>+</sup>) T lymphocyte responses by an in vitro ICC production. Fresh PBMC were stimulated in absence (*left panels*) or presence (*right panels*) of overlapping Gag peptide pool and stained as described in *Materials and Methods*. Lymphocyte population was first gated for CD3<sup>+</sup> lymphocytes, and the CD69<sup>+</sup> TNF- $\alpha$ <sup>+</sup> cell population was enumerated separately in CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) populations (relative percentages of CD69<sup>+</sup> TNF- $\alpha$ <sup>+</sup> cells are given in *upper right corners* of acquisition graphs). *f*, Relative percentages of blood CD8<sup>+</sup> T lymphocytes specific for SIV proteins at days 13 and 42 postchallenge detected by ICC assay. Relative percentages of CD69<sup>+</sup> TNF- $\alpha$ <sup>+</sup> cells of total CD3<sup>+</sup>CD8<sup>+</sup> T cells are represented (values obtained in unstimulated controls are subtracted).

codominant epitopes is predominant in Mamu-A\*01-positive macaques during primary infection (35).

To confirm the presence of secondary immune responses to Gag and Env Ags by an independent functional assay, we monitored the response to these Ags by an ELISPOT assay measuring the IFN- $\gamma$  production following an *in vitro* stimulation with the overlapping peptide pools, an assay that does not distinguish between the CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte responses. At day 9 and 13 postchallenge, animals in both vaccinated groups mounted higher responses to both Gag and Env Ags than the animals in the control group. Thus, this assay independently confirmed the occurrence of a secondary response in the vaccinated animals (Fig. 3).

#### Postchallenge virus-specific T lymphocyte immune responses correlated with viremia containment

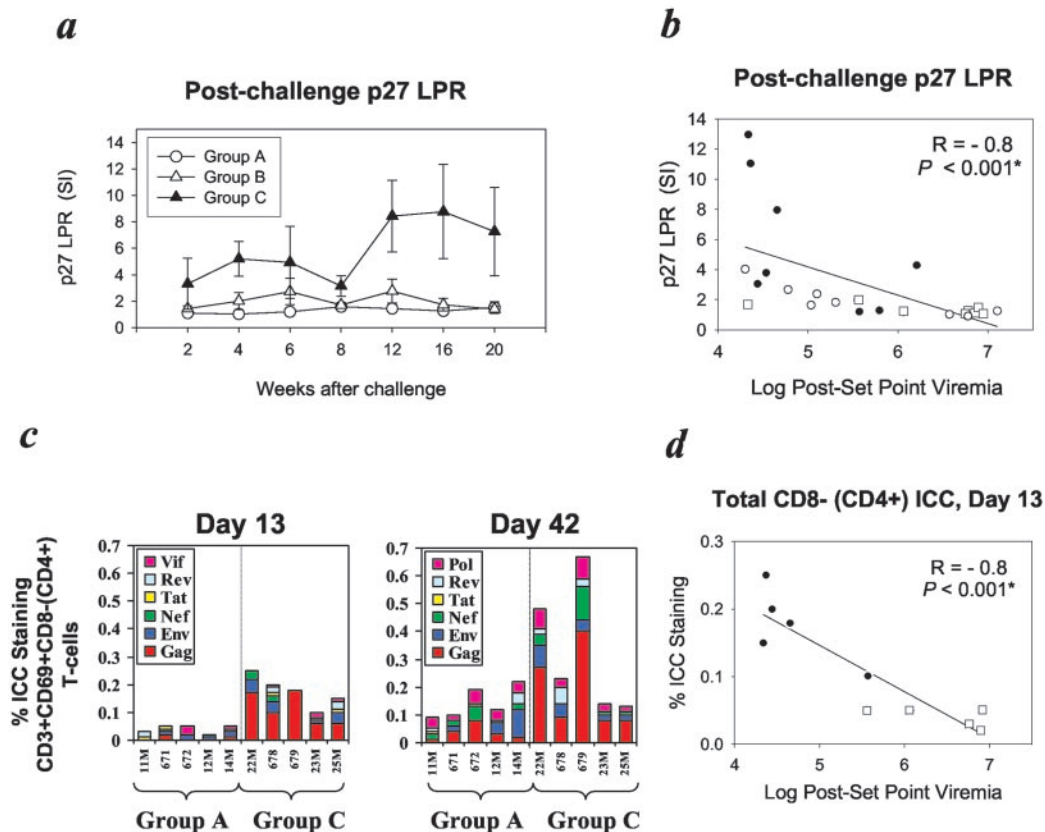
The postchallenge LPRs to Gag p27 protein were significantly higher in group C animals than in control group animals (Fig. 4a,  $p = 0.01$ ) and correlated with post-set point viremia containment (Fig. 4b,  $p < 0.001$ ). Notably, the p27 LPRs were highest in macaque 25 M (maximum level stimulation index = 28), which had detectable plasma virus at only a single time point (Fig. 1b). Few or no LPRs to gp120 Env Ag were detected following the viral challenge.

Both the Gag-specific and the total SIV-specific CD4<sup>+</sup> Th lymphocyte populations measured by the ICC assay at day 13 of acute viremia were significantly higher in the DNA/NYVAC-SIV-*gpe*-immunized group C animals compared with the control animals ( $p = 0.008$  in both cases, Fig. 4c). Interestingly, both the Gag-

specific and the total SIV CD4<sup>+</sup> T lymphocyte responses detected in the infected animals at day 13 postchallenge inversely correlated with the post-set point viremia levels ( $p = 0.02$  and  $< 0.001$ , respectively) (Fig. 4d; Table I). In contrast, the correlation between the level of Gag-specific or total SIV CD8<sup>+</sup> T lymphocyte responses at the peak of viremia and the post-set point containment of viremia did not reach statistical significance ( $p = 0.2$  and  $0.1$ , respectively) (Fig. 2f) (Table I).

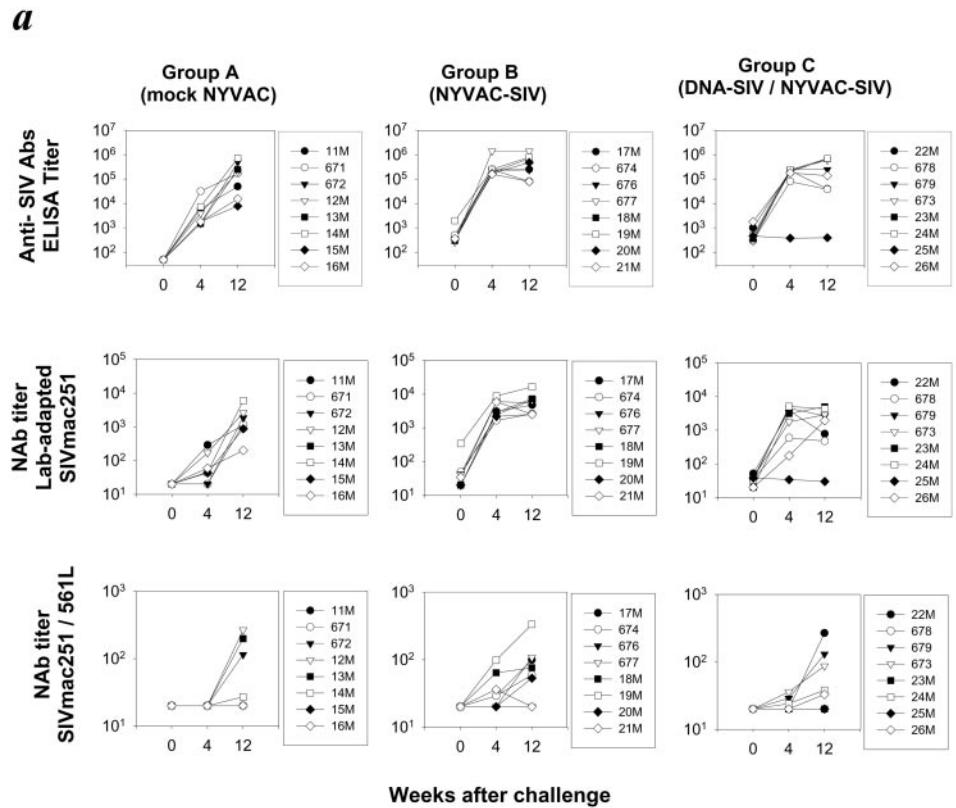
#### Viremia containment did not correlate with the level of neutralizing Abs to the primary SIV<sub>mac251</sub>/561L challenge stock

Mucosal exposure to SIV<sub>mac251</sub> induced a rapid increase in the titers of SIV<sub>mac251</sub>-binding Abs as well as neutralizing Abs to the lab-adapted SIV<sub>mac251</sub> or the primary isolate SIV<sub>mac251</sub>/561L (Fig. 5). At 4 wk after challenge, animals in both vaccinated groups exhibited higher titers of SIV-specific binding Abs ( $p = 0.001$  for group A vs B, and  $p = 0.01$  for group A vs C) and neutralizing Abs to the lab-adapted SIV<sub>mac251</sub> ( $p = 0.001$  for group A vs B, and  $p = 0.007$  for group A vs C) than animals in the control group. In contrast, the level of neutralizing Abs to the primary SIV<sub>mac251</sub>/561L challenge stock was significantly higher only in group B, but not C ( $p = 0.04$  for group A vs B, and  $p = 0.2$  for group A vs C). There was no correlation between prechallenge gp120-specific LPR and the level of SIV<sub>mac251</sub>-binding or -neutralizing Abs at any time point (Table I). No significant negative or positive correlation was found between the level of post-set point viremia containment and the titer of SIV<sub>mac251</sub>-binding



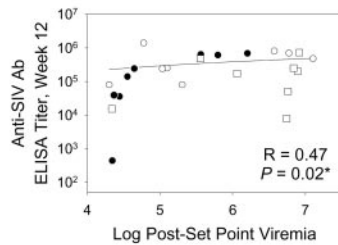
**FIGURE 4.** Postchallenge CD4<sup>+</sup> T lymphocyte responses correlate with the containment of viremia. *a*, Group mean postchallenge LPR to Gag p27 Ag with SEs indicated by bars. *b*, Correlation of average postchallenge LPR to p27 Gag with the average level of post-set point viremia. □, Group A animals; ○, group B animals; ●, group C animals. *c*, Relative percentages of CD3<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) T lymphocytes specific for SIV proteins in blood of challenged animals at days 13 and 42 postchallenge quantitated by ICC assay. Relative percentages of CD69<sup>+</sup> TNF- $\alpha$ <sup>+</sup> cells of total CD3<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) T cells are represented. *d*, Correlation of total frequencies of SIV-specific CD3<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) T lymphocytes quantitated by ICC assay at day 13 postchallenge with the average level of post-set point viremia. □, Group A animals; ●, group C animals.



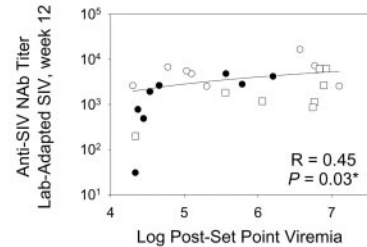


**FIGURE 5.** Anti-SIVmac251-binding and -neutralizing Abs following the viral challenge. *a*, End-point titers of the anti-SIV-binding Abs (top panels) were defined as the reciprocal of the highest sera dilution that gave an optical absorbency at least 2 SDs greater than average values obtained with negative control sera. Neutralizing Ab titers represent the reciprocal plasma dilution at which 50% of the target cells were protected from killing induced by lab-adapted SIVmac251 (middle panels) or challenge stock SIVmac251/561L (bottom panels) virus. *b* and *c*, Positive correlation between the level of anti-SIVmac251-binding Abs determined by ELISA (*b*) or neutralizing Abs to the lab-adapted SIVmac251 (*c*) at 12 wk postinfection and the average level of post-set point viremia (6–24 wk postchallenge). □, Group A animals; ○, group B animals; ●, group C animals.

**b**



**c**



or -neutralizing Abs against the lab-adapted SIVmac251 at 4 wk postchallenge; however, at 12 wk postchallenge, a significant positive correlation was observed between both binding and neutralizing Abs and viremia level ( $p = 0.02$  and  $0.03$ , respectively; Table I; Fig. 5, *b* and *c*). The level of neutralizing Ab to the primary isolate SIVmac251/561L challenge stock virus did not significantly correlate with viremia containment at either time point (Table I). Moreover, animal 25 M that controlled viremia below the level of detection exhibited only low levels of neutralizing Abs to the lab-adapted virus and no detectable neutralizing Ab against the challenge stock virus. Thus, although the immunization with NYVAC-SIV-*gpe* primes for anti-SIVmac251 humoral responses following the mucosal challenge, the increase in the Ab titers did not inversely correlate with viremia containment.

## Discussion

We report in this study that the immunization with NYVAC-SIV-*gpe* alone or in combination with DNA-SIV-*gag*, *env* priming induced virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses that were expanded following mucosal challenge exposure to the highly pathogenic SIVmac251. The secondary immune response correlated with viremia containment during both the acute and chronic

phase of infection. Although both groups of vaccinated macaques mounted secondary immune responses, the reduction of viremia was significantly higher in the DNA-primed macaques, suggesting quantitative and, possibly, qualitative differences between the two modes of vaccination.

The ability of the DNA-prime/live vector-boost vaccine regimen to confer protection against retroviral infection has been investigated in previous studies using SHIV-89.6P or HIV-1 virus infection in macaques (1–4). However, none of these studies have clearly demonstrated a significant advantage of the DNA-prime protocol over the immunization with live vector alone. In this study, we demonstrated that DNA priming significantly improves the protective immunity induced by a live vector. Although sterilizing protection from infection was not observed with this vaccine regimen, a similar long-term containment of viremia, if achieved in humans, could, theoretically, decrease the rate of HIV-1 transmission and limit the spread of the HIV epidemic (36). Although the mechanism underlying the improvement of protective immunity conferred by DNA priming remains unclear, possible explanations include: 1) recruitment of T cells with high affinity TCRs due to a more prolonged low level expression of Ag in DNA-transfected cells (37); 2) absence of

immunogen competition between the transgene product and vector-encoded Ags (38–40); 3) decreased ability of the dendritic cells infected with the vaccinia-based virus to present Ags to CD4<sup>+</sup> T lymphocytes due to their interference with dendritic cell maturation (41, 42).

Previous studies clearly demonstrated a major role of CD8<sup>+</sup> T cells in containing viral replication (43–46). Indeed, in this study, we demonstrated a correlation between the ability to contain viral replication and the frequencies of Gag<sub>CM9</sub> epitope-specific CTLs induced by vaccination and expanded during acute infection. However, development and maintenance of effective CTL pools require CD4<sup>+</sup> T lymphocyte function and an appropriate balance of cytokines (47–49). Importantly, our study shows a significant positive correlation between the levels of prechallenge p27-specific LPR and peak prechallenge levels of Gag<sub>CM9</sub>-specific CD8<sup>+</sup> T cells ( $p = 0.04$ , Table I). In fact, in this study, we provide evidence that the induction of high frequencies of virus-specific CD4<sup>+</sup> T lymphocytes by vaccination might be key to achieving control of infection, because: 1) priming with DNA markedly increased LPRs to both Gag and Env proteins following a subsequent immunization with NYVAC-SIV-*gpe* (23); 2) the post-set point containment of viremia correlated with vaccine-induced as well as postchallenge Gag-specific LPRs; 3) containment of viremia correlated with the frequencies of SIV-specific CD4<sup>+</sup>, but not CD8<sup>+</sup> T lymphocytes during acute infection; 4) both the pre- and postchallenge levels of Gag<sub>CM9</sub>-specific CTLs detected by tetramer staining as well as the total CD8<sup>+</sup> T lymphocyte response to Gag Ag detected by ICC assay at the peak of acute viremia (day 13) directly correlated to the levels of vaccine-induced LPRs to Gag Ag (Table I). In addition, macaque 25 M that was infected, but did not have detectable plasma virus during primary viremia, had high levels of both pre- and postchallenge Gag-specific LPRs (Figs. 1*d* and 4*b*), as well as a high frequency of Gag- and Env-specific CD4<sup>+</sup>, but not CD8<sup>+</sup>, T lymphocytes (Figs. 2*f* and 4*c*).

CD4<sup>+</sup> T cells are the primary targets of HIV-1 infection, and the progressive loss of these cells is associated with AIDS. HIV-1 appears to infect activated HIV-1-specific CD4<sup>+</sup> T lymphocytes during their transition from naive to memory phenotype (50, 51). It appears plausible that the DNA-prime/live vector-boost vaccine regimen induces virus-specific CD4<sup>+</sup> T lymphocytes at sufficient frequency to counter the loss of these cells during the initial burst of virus replication. Preservation of CD4<sup>+</sup> T cells may provide sufficient helper activity for APCs and maintain CTL function. Recent data demonstrate that adoptive transfer of naive autologous CD4<sup>+</sup> T cells to chronically SIV<sub>mac</sub>-infected rhesus macaques promoted virus-specific CTL responses and induced long-term control of virus replication, and further support a key role of CD4<sup>+</sup> Th cells in the control of infection (21). Moreover, virus-specific CD4<sup>+</sup> responses are vigorous in macaques infected with live attenuated SIV that are protected against heterologous challenge (52). Interestingly, also in SIV<sub>mac251</sub>-infected macaques treated with antiretroviral therapy during primary (20) or longstanding (54) infection, an inverse correlation was also observed between the extent of p27 LPR induced by immunization with NYVAC-SIV-*gpe* and viremia containment following drug withdrawal. Finally, virus-specific Th responses were shown to be associated with the induction of optimal CTL responses in both mucosal and systemic tissues (53). Together, these data suggest that virus-specific CD4<sup>+</sup> Th lymphocyte responses are important immune correlates of viremia containment in SIV/HIV-1 infection. It remains to be elucidated whether these cells exhibit a direct antiviral activity or provide help to the effector CD8<sup>+</sup> T cell population.

In this work, we demonstrate that although both NYVAC-SIV-*gpe* and DNA/NYVAC-SIV-*gpe* vaccine candidates prime for anti-

SIV<sub>mac251</sub> humoral responses following the mucosal challenge with SIV<sub>mac251</sub>, the levels of pre- or postchallenge SIV<sub>mac251</sub>-specific Abs do not inversely correlate with viremia containment. In contrast, we observed a positive correlation between the levels of virus-specific Abs at 12 wk after challenge and viremia following the set point of infection. It is possible that the presence of a positive correlation is a mere reflection of an Ag-driven expansion of this virus-specific immune response.

In conclusion, the DNA-prime NYVAC-SIV-*gpe*-boost vaccine regimen used in this study primed for secondary immune responses of both the CD4<sup>+</sup> and CD8<sup>+</sup> T cells that, in concert, contributed to viremia containment following a mucosal challenge exposure to highly pathogenic SIV<sub>mac251</sub> virus. In addition, the data presented in this work provide compelling evidence on the importance of virus-specific CD4<sup>+</sup> T lymphocyte responses in the containment of viral replication. Vaccination with HIV-1-based DNA-prime/NYVAC-boost vaccine regimen may, therefore, be an effective way to decrease the rate of HIV-1 transmission and limit the spread of HIV infection.

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