

## ALVAC-SIV-*gag-pol-env*-Based Vaccination and Macaque Major Histocompatibility Complex Class I (A\*01) Delay Simian Immunodeficiency Virus SIV<sub>mac</sub>-Induced Immunodeficiency

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**T-cell-mediated immune effector mechanisms play an important role in the containment of human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) replication after infection. Both vaccination- and infection-induced T-cell responses are dependent on the host major histocompatibility complex classes I and II (MHC-I and MHC-II) antigens. Here we report that both inherent, host-dependent immune responses to SIV<sub>mac251</sub> infection and vaccination-induced immune responses to viral antigens were able to reduce virus replication and/or CD4<sup>+</sup> T-cell loss. Both the presence of the MHC-I Mamu-A\*01 genotype and vaccination of rhesus macaques with ALVAC-SIV-*gag-pol-env* (ALVAC-SIV-*gpe*) contributed to the restriction of SIV<sub>mac251</sub> replication during primary infection, preservation of CD4<sup>+</sup> T cells, and delayed disease progression following intrarectal challenge exposure of the animals to SIV<sub>mac251</sub> (561). ALVAC-SIV-*gpe* immunization induced cytotoxic T-lymphocyte (CTL) responses cumulatively in 67% of the immunized animals. Following viral challenge, a significant secondary virus-specific CD8<sup>+</sup> T-cell response was observed in the vaccinated macaques. In the same immunized macaques, a decrease in virus load during primary infection ( $P = 0.0078$ ) and protection from CD4 loss during both acute and chronic phases of infection ( $P = 0.0099$  and  $P = 0.03$ , respectively) were observed. A trend for enhanced survival of the vaccinated macaques was also observed. Neither boosting the ALVAC-SIV-*gpe* with gp120 immunizations nor administering the vaccine by the combination of mucosal and systemic immunization routes increased significantly the protective effect of the ALVAC-SIV-*gpe* vaccine. While assessing the role of MHC-I Mamu-A\*01 alone in the restriction of viremia following challenge of nonvaccinated animals with other SIV isolates, we observed that the virus load was not significantly lower in Mamu-A\*01-positive macaques following intravenous challenge with either SIV<sub>mac251</sub> (561) or SIV<sub>SME660</sub>. However, a significant delay in CD4<sup>+</sup> T-cell loss was observed in Mamu-A\*01-positive macaques in each group. Of interest, in the case of intravenous or intrarectal challenge with the chimeric SIV/HIV strains SHIV<sub>89.6P</sub> or SHIV<sub>KU2</sub>, respectively, MHC-I Mamu-A\*01-positive macaques did not significantly restrict primary viremia. The finding of the protective effect of the Mamu-A\*01 molecule parallels the protective effect of the B\*5701 HLA allele in HIV-1-infected humans and needs to be accounted for in the evaluation of vaccine efficacy against SIV challenge models.**

The rate of human immunodeficiency virus type 1 (HIV-1) infection in developing countries has significantly increased in the last few years, and there is therefore an urgent need for the development of an effective vaccine. Studies in animal models have demonstrated the potential role of HIV-based neutralizing antibodies (Ab) in protecting against HIV infection (4, 34). The induction of neutralizing Ab against primary isolates by various vaccine modalities, however, has proven to be difficult, and restriction of viral replication by cell-mediated immune

effector mechanisms appears to be a more realistic goal at present. The contribution of cell-mediated immune responses in controlling HIV-1 replication has been inferred in both acute and chronic HIV-1 infections (8, 27, 40, 42, 47) and clearly demonstrated in the simian immunodeficiency virus macaque (SIV<sub>mac</sub>) model (22, 35, 52).

Poxvirus-based vaccine candidates with various degrees of attenuation are known to induce cell-mediated immune responses and have been shown to prevent infection following challenge exposure to viruses with low virulence, such as some strains of HIV-2 (1, 3, 15) or SIV<sub>mac</sub> (21). They were also found to reduce viral burden following challenge exposure to highly pathogenic SIV<sub>mac</sub> isolates (7, 17, 41, 53). In addition,

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among the poxvirus vaccine candidates, NYVAC and ALVAC have also been demonstrated to be able to induce virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in SIV-infected macaques treated with antiretroviral therapy (18; our unpublished results).

ALVAC (canarypox virus)-based immunogens have been extensively evaluated as veterinary and human vaccine candidates (44) (unpublished results) with three such vaccines being registered with regulatory agencies. These are ALVAC-rabies and ALVAC-feline leukemia virus vaccines for cats and ALVAC-canine distemper virus vaccine for dogs. ALVAC-based HIV-1 vaccine candidates have been tested in more than 1,200 human volunteers and have been shown to be safe and immunogenic (11, 45). Preventive immunization of macaques with a canarypox vector-based HIV-2 immunogen was found to protect macaques from a nonpathogenic HIV-2 challenge (15). The relative efficacy of this vaccine modality, however, has not been assessed previously in the highly pathogenic SIV<sub>mac251</sub> macaque model, in which disease progression and survival can be evaluated.

The usefulness of the SIV<sub>mac251</sub> model in the evaluation of vaccine immunogenicity has been further enhanced by knowledge of macaque major histocompatibility complex (MHC) Mamu-A\*01 status and SIV-specific epitopes restricted by this allelic form (2, 28–30). A study was therefore designed to assess whether immunization with an ALVAC-based vaccine candidate expressing the SIV<sub>mac251</sub> Gag, Pol, and Env components and subsequent boosting with subunit gp120 boost could confer immunity and prevent or contain SIV<sub>mac251</sub> replication following a mucosal exposure to SIV<sub>mac251</sub>. The results indicate that vaccination with ALVAC-SIV-*gpe* modified significantly the natural course of SIV<sub>mac251</sub> (561) infection in Mamu-A\*01-negative macaques (i.e., delayed the CD4<sup>+</sup> T-cell loss) and that some Mamu-A\*01-positive macaques naturally controlled viral replication. The MHC class I (MHC-I) Mamu-A\*01 effect was also investigated following intravenous challenge with SIV<sub>mac251</sub> (561) and SIV<sub>SME660</sub> as well as with two simian/human immunodeficiency virus (SHIV) strains. The results indicate that the route of challenge exposure to SIV isolates influences the natural restriction of viremia in Mamu-A\*01-positive animals regardless of the strain used and that in macaques infected with two independent SHIV isolates viremia restriction does not occur regardless of the challenge route.

**MATERIALS AND METHODS**

**Vaccines, immunization protocol, and challenge virus stock.** Sixty-four macaques were used in this study. The ALVAC-SIV-*gpe* (vcp180) was engineered to express the *gag*, *pol*, and *env* genes of SIV<sub>mac251(K6W)</sub> (14) from the I3L and the H6 promoters (43). The H6 *env* and the I3L *gag* and *pol* cassettes were inserted in the ALVAC C3 locus in a head-to-head (5'-to-5') configuration. Prior to amplification and purification of the vcp180 virus, the expression of the SIV<sub>mac251</sub> genes was assessed in chicken embryo fibroblasts (data not shown). Groups A and B received 10<sup>8</sup> PFU of the ALVAC-SIV-*gpe* vaccine candidate by the intramuscular route at weeks 0, 4, 26, 52, and 113 or 143, and only animals in group B were inoculated intramuscularly with gp120 at weeks 26, 52, and 113 or 143 (Fig. 1). Groups C and D received 10<sup>8</sup> PFU of ALVAC-SIV-*gpe* gp160 at the same intervals as groups A and B by the intramuscular and, in addition, the intrarectal and intranasal routes (Fig. 1). Group D also received SIV gp120 (300 μg) adjuvanted in QS-21 (100 μg) at the same time intervals as group B (Fig. 1). The first control group received 10<sup>8</sup> PFU of ALVAC parental virus by the intramuscular route (group E), and the second was constituted of naive animals

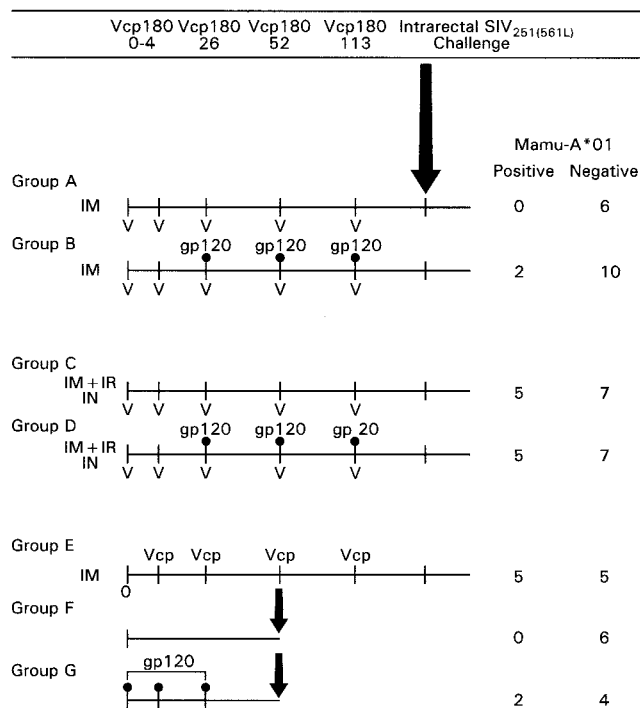


FIG. 1. Schematic representation of the immunization regimen. IM, intramuscular route; IR, intrarectal route; IN, intranasal route; V, vcp180 (ALVAC-SIV-*gpe*), 10<sup>8</sup> PFU; vcp, empty vector control (ALVAC), 10<sup>8</sup> PFU; ●, gp120 (300 μg) in QS-21 (100 μg). The numbers on the right indicate the number of Mamu-A\*01-positive and Mamu-A\*01-negative macaques in each group. The long black arrow signifies the time (week 117 or 147) of intrarectal challenge with SIV<sub>mac251</sub> stock 561.

(group F). Group G received inoculations of gp120 (300 μg) in QS-21 adjuvant (100 μg) at weeks 0, 8, and 55. The SIV gp120 used for immunization was purified from the serum-free culture supernatant of SIV<sub>mac251</sub> chronically infected Hut 78 cells by immunoaffinity column chromatography using anti-gp120 Ab as described previously (25). The macaques were challenged at week 117 of 147; animals were challenged intrarectally with 30 mucosal infectious doses of the SIV<sub>mac251</sub> (561) isolate. The SIV<sub>mac251</sub> challenge stock was prepared by culturing phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from a Mamu-A\*01-positive infected macaque (561L) exposed to SIV<sub>mac251</sub> by the vaginal route. The SIV challenge stock [SIV<sub>mac251</sub> (561)] was titered in vivo in rhesus macaques by inoculating six animals with different dilutions of virus stock via the rectal route. Since six of six animals inoculated with the virus (0.5 ml diluted to 1.5 ml with RPMI medium) became infected, as evidenced by high plasma viremia and a drop in CD4 counts, this dose of virus was selected for all challenge studies.

**SHIV<sub>KU2</sub> challenge virus stock.** The SHIV<sub>KU2</sub> challenge stock (23) was prepared by culturing PHA-activated PBMC from an infected macaque that was inoculated intravenously with SHIV<sub>KU2</sub>. The SHIV<sub>KU2</sub> challenge stock was titered in vivo in rhesus macaques by rectal inoculation of animals with different dilutions of virus stock. Since six of six animals inoculated with the virus (0.5 ml diluted to 1 ml with RPMI medium) became infected, as evidenced by high plasma viremia, this dose of virus was selected for all challenge studies.

**Immunological assays.** Serum samples were tested for SIV-specific Ab responses using an enzyme-linked immunosorbent assay (ELISA) described elsewhere (7). Serum titers were determined as the highest dilutions of immune serum producing ELISA values (A<sub>450</sub>) greater than or equal to two times the binding detected with a corresponding dilution of preimmune serum.

To assess SIV-specific serum-neutralizing activity, two types of assays were conducted with sera from the vaccinated animals. In the first assay, sera were tested for their ability to neutralize a T-cell-line-adapted stock of SIV<sub>mac251</sub> grown in H9 cells and assayed in CEMx174 cells as described previously (38). In the second assay, neutralization of the challenge stock of SIV<sub>mac251</sub> (561) was examined in PHA-activated human PBMC by measuring a reduction in viral p27

TABLE 1. Cytotoxic response to Gag and Env in the immunized animals

ALVAC-SIV- <i>gpe</i> (vcp180) vacci- nation route <sup>a</sup>	gp120 boost <sup>b</sup>	Animal no. <sup>c</sup>	% percent CTL activity <sup>d</sup> at week:					
			28		54		115	
			Env	Gag	Env	Gag	Env	Gag
IM	—	428	10	— <sup>e</sup>	9	—	32	—
IM	—	398	12	—	16	—	—	—
IM	—	411	11	—	—	—	28	—
IM	—	412	—	—	—	—	25	—
IM	—	397	—	—	—	—	—	—
IM	—	423	—	—	—	—	—	—
IM	+	402	22	—	40	—	42	26
IM	+	401	10	—	12	—	32	—
IM	+	416	12	—	23	—	14	—
IM	+	415	17	—	22	—	—	—
IM	+	<b>429</b>	13	—	—	—	—	26
IM	+	414	18	—	—	—	—	—
IM, IR, IN	—	<b>400</b>	17	—	47	—	28	—
IM, IR, IN	—	410	10	—	—	—	—	—
IM, IR, IN	—	399	—	—	—	—	—	—
IM, IR, IN	—	413	—	—	—	—	—	—
IM, IR, IN	—	<b>424</b>	—	—	—	—	—	—
IM, IR, IN	—	435	—	—	—	—	—	—
IM, IR, IN	+	<b>409</b>	22	—	—	—	33	—
IM, IR, IN	+	417	10	—	—	—	—	—
IM, IR, IN	+	<b>418</b>	—	—	—	—	19	—
IM, IR, IN	+	430	ND <sup>f</sup>	ND	11	—	—	—
IM, IR, IN	+	<b>403</b>	—	—	—	—	—	—
IM, IR, IN	+	404	—	—	—	—	—	—

<sup>a</sup> IM, intramuscular; IR, intrarectal; IN, intranasal.

<sup>b</sup> gp120 was given intramuscularly in QS-21 adjuvant.

<sup>c</sup> Boldface numbers, Mamu-A\*01-positive animals.

<sup>d</sup> Percentage of cytolytic activity at an effector-to-target ratio of 20:1; cumulative CTL response: 16 of 24 (67%).

<sup>e</sup> —, no cytotoxic activity.

<sup>f</sup> ND, not determined.

Gag antigen synthesis (31). CD4<sup>+</sup> T-cell count in the PBMC of challenged animals was determined by standard flow-cytometric analyses (FAST Systems, Inc., Gaithersburg, Md.).

**CTL assay and tetramer staining.** PBMC ( $8 \times 10^6$ ) from macaques were cultivated in vitro with paraformaldehyde-fixed, autologous B-lymphoblastoid cell lines (B-LCL) infected with vaccinia virus encoding SIV Env and SIV Gag components. On day 3 of culture, 20 U of recombinant human interleukin-2 per ml was added to the cultures. On day 12 of culture, the lymphocytes were centrifuged over a Ficoll-diatrizoate gradient and assessed as effector cells in a standard <sup>51</sup>Cr-release cytolytic assay. Target cells were B-LCL ( $10^6$ ) cultured overnight with vaccinia virus encoding SIV Env, SIV Gag, or control antigen at a multiplicity of infection of 10 PFU/cell. B-LCL were then washed and labeled with 100  $\mu$ Ci of sodium <sup>51</sup>chromate for 1.5 h. After being washed,  $10^4$  target cells were added per well in 96-well U-bottom plates in 100- $\mu$ l volumes. Effector cells were added in another 100- $\mu$ l volume at various concentrations to give effector-to-target ratios of 20:1, 10:1, 5:1, and 2.5:1. Plates were incubated at 37°C for 4 h. Fifty microliters of supernatant was transferred to counting plates and 200  $\mu$ l of scintillation fluid was added and analyzed in a Wallac 1450 MicroBeta liquid scintillation counter. Specific release was calculated according to the following formula: (experimental release – spontaneous release)/(100% release – spontaneous release)  $\times$  100 (Table 1). The Mamu-A\*01-positive rhesus monkeys were evaluated for p11C-specific cytotoxic T-lymphocytes (CTLs) using Mamu-A\*01/p11C tetramer staining of unstimulated peripheral blood CD8<sup>+</sup> T lymphocytes. Soluble tetrameric Mamu-A\*01/peptide complexes were prepared, and 1  $\mu$ g of phycoerythrin-labeled tetrameric Mamu-A\*01/peptide complexes was used in conjunction with fluorescein isothiocyanate-labeled anti-human CD8 $\alpha$  (Leu2a; Becton-Dickinson, San Diego, Calif.), energy-coupled dye-labeled anti-human CD8 $\alpha$  $\beta$  (2S78-5H7; Beckman Coulter, Fullerton, Calif.), and allophycocyanin-labeled anti-rhesus monkey CD3 (FN18) monoclonal Ab to stain p11C-specific CD8<sup>+</sup> T cells. One hundred microliters of whole blood from the vaccinated monkeys was directly stained with these reagents, lysed, washed, and fixed.

**Virological assay.** Animals were bled periodically following challenge, and viral load in plasma was assessed using a nucleic acid sequence-based amplification assay to quantify SIV RNA (48). In addition, PBMC collected from animals 21 days following virus challenge were subjected to quantitative virus isolation by coculturing with CEMx174 cells to confirm virus transmission.

## RESULTS

**Study design.** The experimental vaccination regimen included seven groups (A through G) of rhesus macaques (Fig. 1). The experimental groups A through D were immunized five times with ALVAC-SIV-*gpe* ( $10^8$  PFU) and, at the time of the last three immunizations, animals enrolled in groups B and D were inoculated simultaneously with native SIV<sub>mac251</sub> gp120. Control animals received either five immunizations with ALVAC vector (group E) or were left naive (group F). Lastly, group G animals received three inoculations with gp120. ALVAC-SIV-*gpe* was administered either by the intramuscular route (groups A and B) or by a combination of the intramuscular, intranasal, and intrarectal routes (groups C and D), whereas gp120 was administered by the intramuscular route, as described in Materials and Methods. A total of 19 Mamu-A\*01-positive animals were included in the study and were distributed among the groups as summarized in Fig. 1.

**Humoral immune response elicited by ALVAC-SIV-*gpe*.** Serum Ab to SIV<sub>mac251</sub> were measured in half of the animals from each group by ELISA using whole disrupted SIV<sub>mac251</sub> virus spiked with purified gp120. Ab titers were negligible after two inoculations with ALVAC-SIV-*gpe* (data not shown). However, after the third immunization (week 26), the Ab titers increased and were highest in groups B and D, which also received the QS-21-adjuvanted gp120 (Fig. 2A). Although the Ab titers declined between immunizations, a steady level of Ab was present throughout the course of vaccination (Fig. 2A). A similar pattern was observed with the rest of the animals.

Both immunoglobulin G (IgG)- and IgA-specific Ab responses to SIV gp120 and p27 antigens were measured by ELISA in mucosal secretions such as rectal wash, saliva, and vaginal wash collected at week 115 in six animals from groups A through E. Neither IgG- nor IgA-specific Ab to SIV p27 were detected in animals from any of the vaccine groups (data not shown). In contrast, serum IgG-specific Ab response to gp120 was detected in most animals from groups B and D boosted with gp120 regardless of the route of administration of ALVAC-SIV-*gpe*. A low level of IgA-specific Ab to gp120 was detected in the saliva or vaginal secretions of two animals from group D only (data not shown).

Neutralizing Ab titers in sera from all ALVAC-vaccinated animals (groups A to D) collected at month 12 and 2 weeks following the final boost were measured against those of the laboratory-adapted SIV<sub>mac251</sub> isolate in CEMx174 cells as well as the primary challenge stock of SIV<sub>mac251</sub>(561) in human PBMC. For animals receiving the gp120 boost (group G), sera collected 2 weeks following the final boost were similarly assayed for neutralizing Ab titers. Neutralizing Ab titers to the laboratory-adapted SIV<sub>mac251</sub> were detected in most animals receiving the vaccine candidate but were highest in animals boosted with the gp120 subunit preparation (Fig. 2B), whereas none of the serum samples neutralized the SIV<sub>mac251</sub>(561) primary challenge stock in a human-PBMC-based assay (titers

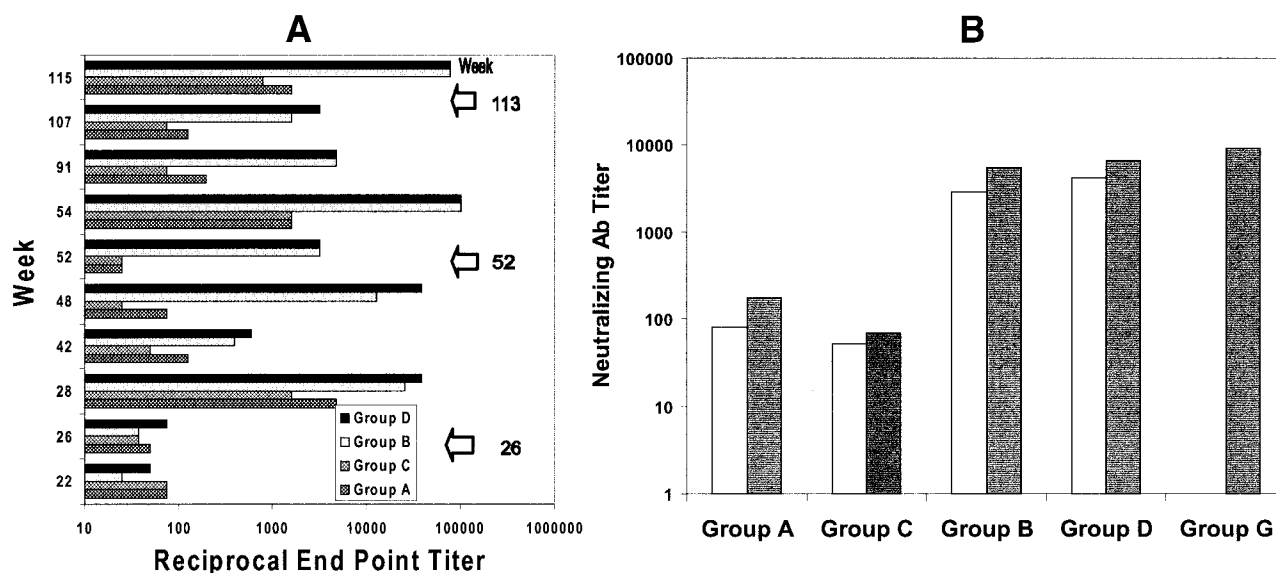


FIG. 2. Humoral response in immunized animals. (A) Median titer values of Ab in prechallenge sera of the immunized macaques. ELISA Ab titers were obtained using a total lysate of SIV<sub>mac251</sub> spiked with purified native gp120. (B) Neutralization Ab titers in sera of ALVAC-SIV-*gpe*-vaccinated macaques. The median neutralizing Ab titer of immunized animals from groups A through D to the laboratory-adapted SIV<sub>mac251</sub> was assessed on CEMx174 cells using sera collected at month 12 (clear bar) and 2 weeks after the final boost (filled bar), whereas for group G animals the assay was conducted using sera collected at 2 weeks after the final boost.

<1:5; 80% reduction in p27 synthesis was considered positive) (data not shown).

**CTL activity induced by ALVAC-SIV-*gpe*.** T-cell-mediated cytolytic activity was assessed in the blood of six of the immunized animals in each group as well as in control animals from group E following the third, fourth, and last immunizations. Cumulatively, 16 of 24 (67%) animals demonstrated an Env-specific CTL activity at at least one time point analyzed. Thirteen animals recognized Env target cells after the third immunization and, of those, nine were also positive following either the fourth or the fifth immunization (Table 1). Overall, 22% of the vaccinees demonstrated CTL activity against either Env or Gag at all times, 42% were positive two times, and 67% were positive at any single point. Among the control animals in group E, only one animal scored positive at a single time point. There was a trend suggesting that the relative frequency of measurement of CTL responses in peripheral blood in animals vaccinated by the systemic route was higher than in animals vaccinated also by the mucosal route (10 of 12 versus 6 of 12, respectively) (Table 1).

A surprising finding was that only 2 of 24 animals had detectable cytolytic activity against the SIV Gag protein, whereas 16 of 24 animals had cytolytic activity against the SIV Env protein. A possible explanation is that CTLs specific for Gag may have existed at a frequency below the level of detection by the assay. In fact, staining with tetramer for the peptide p11C epitope following specific peptide stimulation in vitro demonstrated that most of the vaccinated Mamu-A\*01-positive animals (Table 1) indeed had memory CTL response to the Gag-immunodominant p11C epitope prior to viral challenge exposure (data not shown).

**Mock-vaccinated Mamu-A\*01-positive macaques naturally restrict SIV<sub>mac251</sub> (561) replication.** Following intrarectal challenge exposure to SIV<sub>mac251</sub> stock 561, all 22 macaques from

groups E, F, and G became viremic and seroconverted to viral antigens. Analysis of the plasma virus load during primary infection, set point, and chronic infection did not reveal significant differences among the groups, as summarized in Table 2. These data demonstrate that neither the gp120 subunit immunization nor the ALVAC mock vaccination influenced the virological outcome. Therefore, all these animals were gathered together to increase the statistical power of the subsequent analysis.

Interestingly, measurement of viral RNA in the Mamu-A\*01-positive and -negative mock-vaccinated macaques revealed that among the Mamu-A\*01-positive macaques four of five controlled viremia at set point up to week 17, as demonstrated for each animal in Fig. 3A. In fact, when the virus load in all the control animals (groups E, F, and G) was stratified according to the Mamu-A\*01 status (15 were Mamu-A\*01 negative and 7 were Mamu-A\*01 positive), statistical analysis

TABLE 2. Comparison of virus load among animals from groups E, F, and G

Immunizations	P value <sup>a</sup> for viremia at:		
	Peak	Set point	3-6 mo
Mamu-A*01-negative macaques gp120-immunized (4) vs ALVAC-immunized (11) or naive	0.10	0.49	0.75
Mamu-A*01-positive macaques gp120-immunized (2) vs ALVAC-immunized (5) or naive	0.57	1.0	0.95
Mamu-A*01-positive macaques vs naive Mamu-A*01-negative control macaques gp120- or ALVAC-immunized (7) vs naive (5)	0.76	0.69	0.064

<sup>a</sup> Calculated from the exact Wilcoxon rank sum tests.

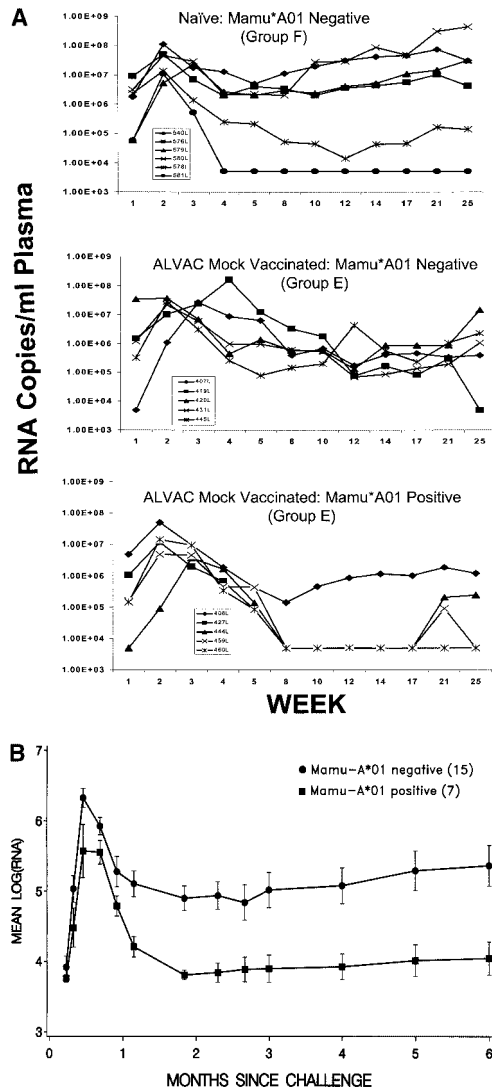


FIG. 3. Levels of viremia in control macaques following SIV<sub>251</sub> challenge. (A) Viral RNA copies/milliliter of plasma over time in each animal from groups E, F, and G (Fig. 1). (B) Mean levels of viral load at each time point in Mamu-A\*01-positive and -negative control macaques.

of the viral-load data demonstrated that in the seven mock-vaccinated Mamu-A\*01-positive animals virus load was significantly lower than in the Mamu-A\*01-negative macaques during primary viremia (0 to 28 days), at set point (2 to 3 months), and thereafter (for primary viremia,  $P = 0.0066$  by the Wilcoxon rank sum test; for set point,  $P = 0.0007$  by the Wilcoxon-Gehan test; for median viremia,  $P = 0.0068$  by the Wilcoxon-Gehan test). In fact, most Mamu-A\*01-positive animals had nondetectable viremia at set point and thereafter, as demonstrated for each animal in Fig. 3A and collectively in Fig. 3B. All together, these data indicate that the Mamu-A\*01-positive macaques naturally restricted replication following intrarectal exposure to SIV<sub>mac251</sub> (561).

**Effect of ALVAC-SIV-*gpe* vaccination in Mamu-A\*01-positive animals.** Because of the natural ability of Mamu-A\*01-positive macaques to control SIV<sub>mac251</sub> (561) intrarectal

infection, the statistical analysis of the relative efficacy of ALVAC-SIV-*gpe* vaccine was assessed independently in the Mamu-A\*01-positive and the Mamu-A\*01-negative animals. Genetic characterization of all animals enrolled in this study demonstrated the presence of 12 Mamu-A\*01-positive animals among the 42 ALVAC-SIV-*gpe*-vaccinated macaques (Fig. 1). Unknown to us at the beginning of the study, the vaccinated Mamu-A\*01-positive animals were interspersed unevenly in the experimental groups A through D (Fig. 1). Since in retrospect we observed a significant containment of viremia in the Mamu-A\*01-positive animals (Fig. 3B), the overall evaluation of relative vaccine efficacy was assessed independently in Mamu-A\*01-positive and -negative vaccinated and control macaques.

In Mamu-A\*01-positive vaccinated and control macaques, the overall difference in viremia during primary infection (0 to

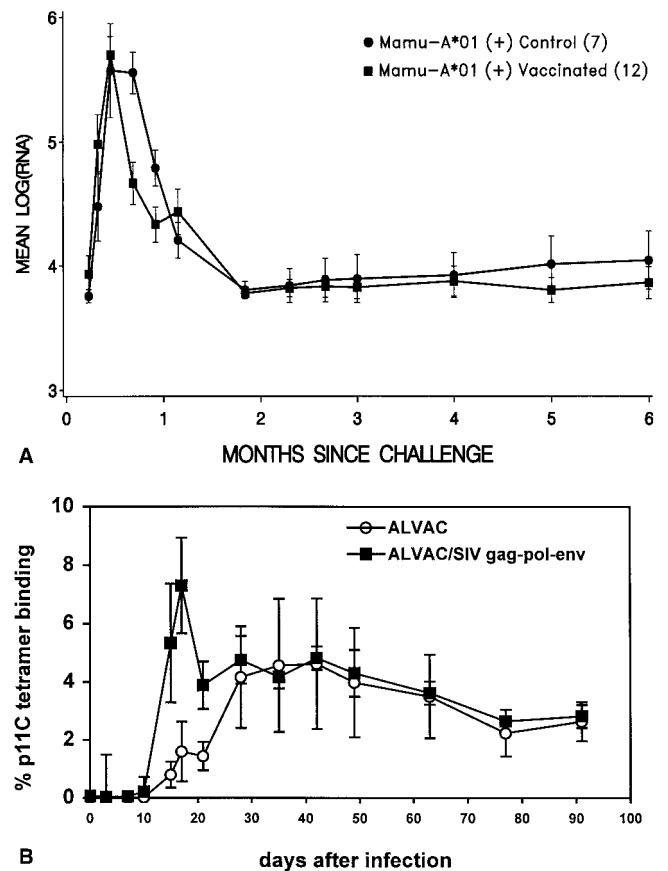


FIG. 4. Viral load and anamnestic response in Mamu-A\*01-positive control and ALVAC-SIV-*gpe*-vaccinated macaques. (A) Average viral load in the mock-vaccinated (●) and ALVAC-SIV-*gpe*-vaccinated (■) Mamu-A\*01-positive macaques. (B) Mean values of the frequency of SIV Gag p11C-tetramer-binding CD8<sup>+</sup> lymphocytes in the whole blood of the control and ALVAC-SIV-*gpe*-immunized Mamu-A\*01-positive rhesus monkeys after challenge with SIV<sub>mac251</sub>. The percent p11C tetramer represents p11C-tetramer-binding CD8 $\alpha\beta^+$  T cells in unstimulated whole blood at each time point tested. Open circles represent the means and standard errors of means (SEMs) of percent p11C-tetramer-binding CD8 $\alpha\beta^+$  T cells of five monkeys that received the control vaccine, and filled squares represent the means and SEMs of percentages of p11C-tetramer-binding CD8 $\alpha\beta^+$  T cells of six monkeys that received the ALVAC-SIV-*gpe* vaccine.

TABLE 3. Statistical analyses of plasma viral RNA load measured at different stages following virus challenge

Groups compared	P value for viral load at:		
	Primary infection (0–28 days) <sup>a</sup>	Set point <sup>b</sup>	3–6 mo
Mamu-A*01-positive macaques, vaccinated (12) vs mock vaccinated (7)	0.54	0.79	0.49
Mamu-A*01-negative macaques, vaccinated (30) vs mock vaccinated (15)	0.0078	0.33	0.95
Intramuscularly immunized macaques (16) vs intramuscularly, intrarectally, intranasally immunized macaques (14)	0.16	0.96	0.94
Mamu-A*01-negative macaques plus gp120 boost (17) vs no gp120 boost (13)	0.8	0.95	0.53

<sup>a</sup> Wilcoxon rank sum test (two-tailed).

<sup>b</sup> Wilcoxon-Gehan test (two-tailed).

28 days) was not significant (Table 3), even though the vaccinated macaques appeared to control viremia faster than the nonvaccinated Mamu-A\*01-positive animals (Fig. 4A). In fact, the quantitation of CD3<sup>+</sup> CD8<sup>+</sup> peptide p11C tetramer response in the blood of animals following challenge exposure demonstrated a faster appearance of this response in the vaccinated animals than in control animals (Fig. 4B), consistent with a secondary response. This difference was found to be significant in the interval from day 13 through day 28 postchallenge ( $P = 0.0075$ ) by the application of repeated-measures analysis of variance to the arc-sine-transformed tetramer data (Fig. 4B). These data further support the importance of cell-mediated immunity in controlling SIV replication, as demonstrated by other studies (22, 29, 35, 52), and indicate that the ability of Mamu-A\*01-positive animals to restrict viral replication may mask the relative efficacy of vaccines. No significant difference in the early CD4<sup>+</sup> T-cell drop was observed between these two groups (data not shown). Accordingly, no deaths were observed in the Mamu-A\*01-positive group regardless of the vaccination status (Fig. 5C).

**ALVAC-SIV-gpe vaccination decreases primary viremia and CD4<sup>+</sup> T-cell loss in Mamu-A\*01-negative macaques.** The effects of ALVAC-SIV-gpe vaccination on viral load and the natural course of SIV<sub>mac251</sub> infection were evaluated separately in the Mamu-A\*01-negative macaques. Of the 30 vaccinated and 15 mock-vaccinated Mamu-A\*01-negative macaques, all became infected except 2 vaccinees. The 30 vaccinated animals experienced lower viremia in primary infection (first 28 days) than the 15 control macaques ( $P = 0.0078$ ) (Table 3). When the peak viremia in the 30 vaccinated animals was compared to that in 11 control macaques (excluding 4 macaques that received gp120), this difference was even more significant ( $P = 0.0034$ ) (Fig. 5A). The set-point viremias in the control and vaccinated macaques did not differ significantly (Table 3), but, remarkably, the analysis of the absolute CD4<sup>+</sup> T-cell count (Fig. 5B) indicated that the 30 vaccinated animals were protected from acute loss of CD4<sup>+</sup> T cells within the first 2 months of infection ( $P = 0.0099$ ) and during the 1-year follow-up ( $P = 0.03$ ). Among the vaccinated Mamu-A\*01-negative macaques, fewer deaths occurred than among

Mamu-A\*01-negative control animals, but this difference did not reach statistical significance (Fig. 5C).

The contribution of boosting with the gp120 subunit preparation and the route of immunization of the ALVAC-SIV-gpe vaccine effect were also analyzed by comparing virus load and CD4<sup>+</sup> T-cell count in Mamu-A\*01-negative animals from groups A and C to those in animals from groups B and D. As demonstrated in Table 2, neither the monomeric gp120 subunit preparation nor the combination of the routes of immunization appeared to significantly contribute to the effect of ALVAC-SIV-gpe vaccination. Accordingly, no significant difference in the CD4<sup>+</sup> T-cell counts was observed between the vaccinated groups of animals (data not shown).

Collectively, these data demonstrate that a decrease in virus load during primary and chronic infection and preservation of CD4<sup>+</sup> T cells occurred in Mamu-A\*01-positive animals regardless of their vaccination status. In Mamu-A\*01-negative animals, however, a vaccine effect was also observed, and those macaques experienced a significantly lower level of viremia during primary infection than did control macaques. This effect was presumably associated with a better preservation of CD4<sup>+</sup> T cells and was associated with fewer deaths in the vaccinated animals than in control animals within 1 year of SIV<sub>mac251</sub> (561) infection.

**Preservation of CD4<sup>+</sup> T cells in Mamu-A\*01-positive macaques following intravenous challenge with either SIV<sub>mac251</sub> (561) or SIV<sub>SME660</sub>.** We further investigated whether the inherent ability of Mamu-A\*01-positive macaques to restrict viral replication observed in our study was dependent on the route of challenge. To do so, virus load and CD4<sup>+</sup> T-cell counts were analyzed for up to 24 weeks postchallenge in 5 Mamu-A\*01-positive and 12 Mamu-A\*01-negative naive macaques previously infected intravenously with the same 561 stock of SIV<sub>mac251</sub>. In addition, to assess whether the Mamu-A\*01 effect could also be extended to other SIV isolates, we retrospectively analyzed the virus load and CD4<sup>+</sup> T-cell count in Mamu-A\*01-positive and -negative control macaques infected previously with SIV<sub>SME660</sub> (41, 53, 54). Analysis of the virus loads in the blood of macaques following intravenous exposure to SIV<sub>mac251</sub> (561) or SIV<sub>SME660</sub> did not reveal a significant reduction in virus load within the first 20 weeks of infection in animals from either group (data not shown). However, longitudinal analysis of the absolute CD4<sup>+</sup> T-cell count during the same period indicated a better preservation of the absolute CD4<sup>+</sup> T-cell count in Mamu-A\*01-positive macaques infected with either virus (Fig. 6). In fact, in animals infected with the SIV<sub>mac251</sub> (561) strain, repeated-measures analysis of variance on the square-root-transformed CD4<sup>+</sup> T-cell count over a 6-month interval revealed that the differences in CD4<sup>+</sup> T-cell counts were significant at the  $P$  level of  $<0.001$  for each of the intervals analyzed (Fig. 6, top panel). Similarly, in macaques inoculated intravenously with SIV<sub>SME660</sub>, the loss in CD4<sup>+</sup> T-cell counts differed significantly between Mamu-A\*01-positive and -negative animals (Fig. 6, bottom panel) and was delayed in Mamu-A\*01-positive macaques (weeks 8, 12, and 16;  $P < 0.052$  for each by the Wilcoxon rank sum test and  $P < 0.0001$  by the repeated-measures analysis of variance). All together, these results need to be accounted for in the evaluation of a vaccine effect when preservation of CD4<sup>+</sup> T cells is included as a parameter of vaccine protection.

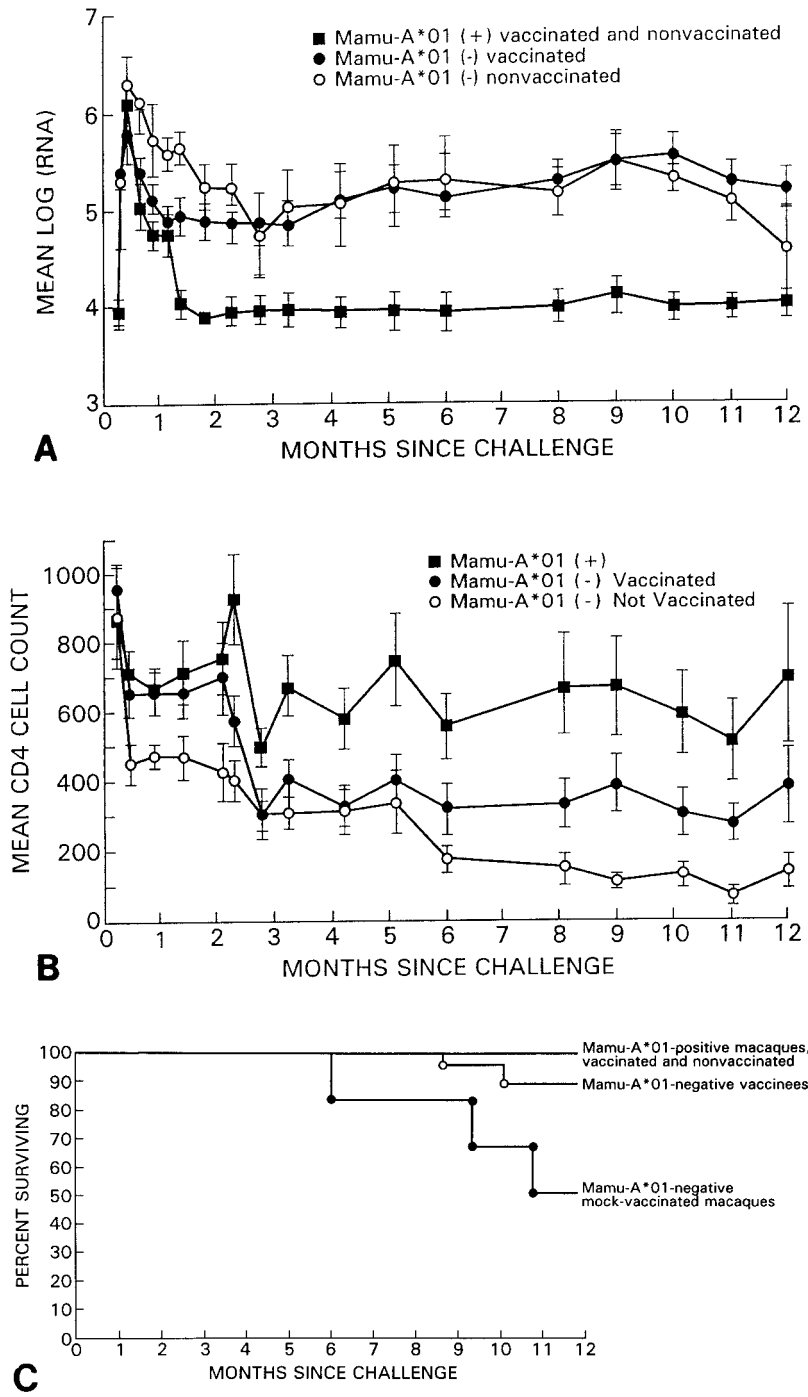


FIG. 5. Virus load, CD4<sup>+</sup> T cell counts, and survival in vaccinated and control Mamu-A\*01-negative macaques. (A) Mean virus load in Mamu-A\*01-negative vaccinated (●), control (○), and all vaccinated and nonvaccinated Mamu-A\*01-positive animals (■). In this analysis, only animals that were monitored for 1 year are included. Therefore, the group of Mamu-A\*01-positive animals included 8 macaques (6 vaccinees and 2 controls), the group of Mamu-A\*01-negative vaccinees included 18 macaques, and the group of Mamu-A\*01-negative control animals included 6 macaques. Peak viral loads during primary infection differed significantly between vaccinated and mock-vaccinated Mamu-A\*01-negative macaques ( $P = 0.011$ ) when analyzed using the Wilcoxon rank sum test. Because of the frequency of left censoring, the Wilcoxon-Gehan test was applied to the median viral load over later intervals. However, in these intervals no significant difference in virus load was observed ( $P = 0.37$  at set point and  $P = 0.97$  thereafter). (B) Mean CD4<sup>+</sup> T-cell count in the animal groups described in the legend to panel A. The same symbols are used as for panel A, and the same animals per group were analyzed. CD4<sup>+</sup> T-cell counts were tested by repeated-measures analysis of variance of the square-root-transformed raw data, and during primary infection a significant difference was observed between vaccinated and control animals ( $P = 0.013$ ). (C) Number of deaths for SIV<sub>mac251</sub>-related disease within 1 year of viral challenge. The animals per group are the same as for panel A. Survival probabilities were estimated by the Kaplan-Meier method and demonstrated a trend of higher survival in vaccinated macaques than in control macaques.

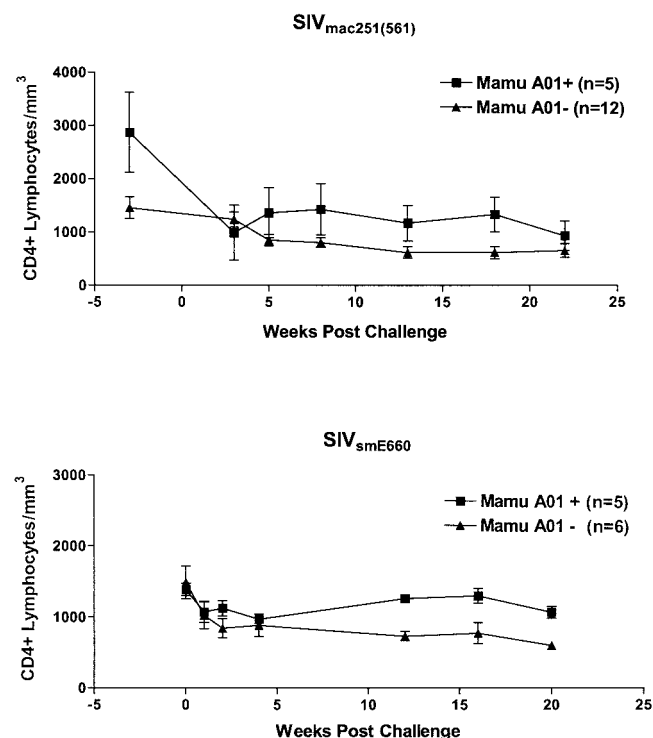


FIG. 6. Analysis of the mean CD4<sup>+</sup> T-cell value in Mamu-A\*01-positive and Mamu-A\*01-negative macaques after intravenous SIV challenge. (Top) Data from five Mamu-A\*01-positive and 12 Mamu-A\*01-negative macaques challenged intravenously with SIV<sub>mac251</sub> (stock 561). (Bottom) Data from five Mamu-A\*01-positive and six Mamu-A\*01-negative macaques challenged intravenously with SIV<sub>SME660</sub>.

**The Mamu-A\*01 effect is not evident following infection with two chimeric SIV/HIV-1 strains.** Other macaque models used in the evaluation of HIV-1 vaccine candidates include the use of chimeric SHIVs. To investigate whether the Mamu-A\*01 molecule could also have a protective effect following SHIV infection, the viremia and CD4<sup>+</sup> T-cell counts of Mamu-A\*01-positive and -negative macaques following challenge with either SHIV<sub>89.6P</sub> by the intravenous route or SHIV<sub>KU2</sub> by the intrarectal route were compared. Viremia levels and CD4<sup>+</sup> T-cell numbers did not differ among Mamu-A\*01-positive and -negative animals following SHIV<sub>89.6P</sub> intravenous challenge (Fig. 7, upper panels) or SHIV<sub>KU2</sub> intrarectal challenge (Fig. 7, lower panels).

## DISCUSSION

In this study, we have demonstrated that prophylactic ALVAC-SIV-*gpe* vaccination of macaques followed by intrarectal challenge exposure with the highly pathogenic SIV<sub>mac251</sub> strain was associated with lower primary viremia, better preservation of CD4<sup>+</sup> T-cell counts during primary and chronic infection, and prolonged survival. The neutralizing Ab response induced by a monomeric gp120 subunit preparation did not contribute to the observed protection from disease, presumably because none of the animals developed an Ab response capable of neutralizing the primary-challenge stock virus. However, vaccination with the ALVAC-SIV-*gpe* induced SIV-specific CD8<sup>+</sup> T-cell virus-specific cytolytic activity in 67%

of the vaccinated animals and was associated with a significant secondary response to the Gag p11C peptide in the Mamu-A\*01-positive macaque following virus challenge exposure. Similarly, the vaccinated Mamu-A\*01-negative macaque did better than control nonvaccinated macaques.

It is to be noted that the viral challenge stock used in this study infected 100% of the control macaques (22 control animals and 6 animals in the titration study). It is quite likely that the infectivity of this stock as well as the size of virus inoculum used during challenge may far exceed that of HIV-1 transmission, as the recently estimated frequency of transmission of HIV-1 in humans appears to be relatively low (46, 50).

The immunogenicity of ALVAC-based HIV vaccine candidates has been extensively studied in human volunteers enrolled in clinical trials of phases I and II, and the immunogenicity of this vaccine in humans mirrors that observed in the macaques studied here (6, 10, 12, 13, 16, 24, 51). However, despite clear evidence of immunogenicity, enthusiasm to proceed to extended efficacy trials has been tempered by incomplete knowledge of vaccine-induced immune parameters and protection against lentivirus exposure. The study presented here indicates that although vaccinated macaques were not protected from infection they did demonstrate significant differences in the virological and clinical outcome following exposure to a pathogenic SIV<sub>mac251</sub> challenge. It should be noted that despite such uncertainties as to whether results obtained in the macaque model can be extrapolated to humans, recent studies seem to indicate that it may be appropriate to do so. For instance, therapeutic intervention in SIV<sub>mac251</sub> primary infection (18, 20, 32, 33, 39, 55) has paralleled closely results obtained in primary HIV-1 infection in humans (49), validating the SIV macaque model. Furthermore, the observation presented in this communication (that Mamu-A\*01-positive macaques are better able to control viral replication than macaques carrying other MHC-I molecules) highlights additional similarities to HIV-1 infection of humans. In HIV-1 infection, the maximum heterogeneity of HLA class I molecules or the single HLA-B\*5701 molecule has been associated with a more benign clinical course (9, 37) whereas HLA A1-B8-DR3, B27, CW7, and B\*35-Cw\*04 molecules are correlated with faster disease progression (9, 26, 36, 56). Mamu-A\*01-positive rhesus macaques appear to develop early robust multiepitope virus-specific CD8<sup>+</sup> T-cell responses (2) (B. R. Mothe et al., submitted for publication), which persist in various compartments (19) and may contribute to their ability to restrict viral replication, as observed in this study, and this further underscores the importance of the breadth of CD8<sup>+</sup> T-cell immune response to the containment of viral replication. Finally, this study indicates that the genetic background of macaques used in preclinical studies of the relative efficacy of the SIV<sub>mac251</sub> model needs to be accounted for in comparative studies. In this context, it is to be noted that upon intravenous challenge the Mamu-A\*01-related protective effect was more evident on the loss of CD4<sup>+</sup> T cells than on viremia. This effect did not appear to be restricted to our viral stock, since it was observed also with the SIV<sub>SME660</sub> strain. Interestingly, however, in other studies in which intravenous transmission of SHIV<sub>89.6P</sub> viruses was assessed in naive Mamu-A\*01-positive and -negative macaques (5) and in our studies following intrarectal challenge with SHIV<sub>KU2</sub>, the Mamu-A\*01 effect was not evident, regard-



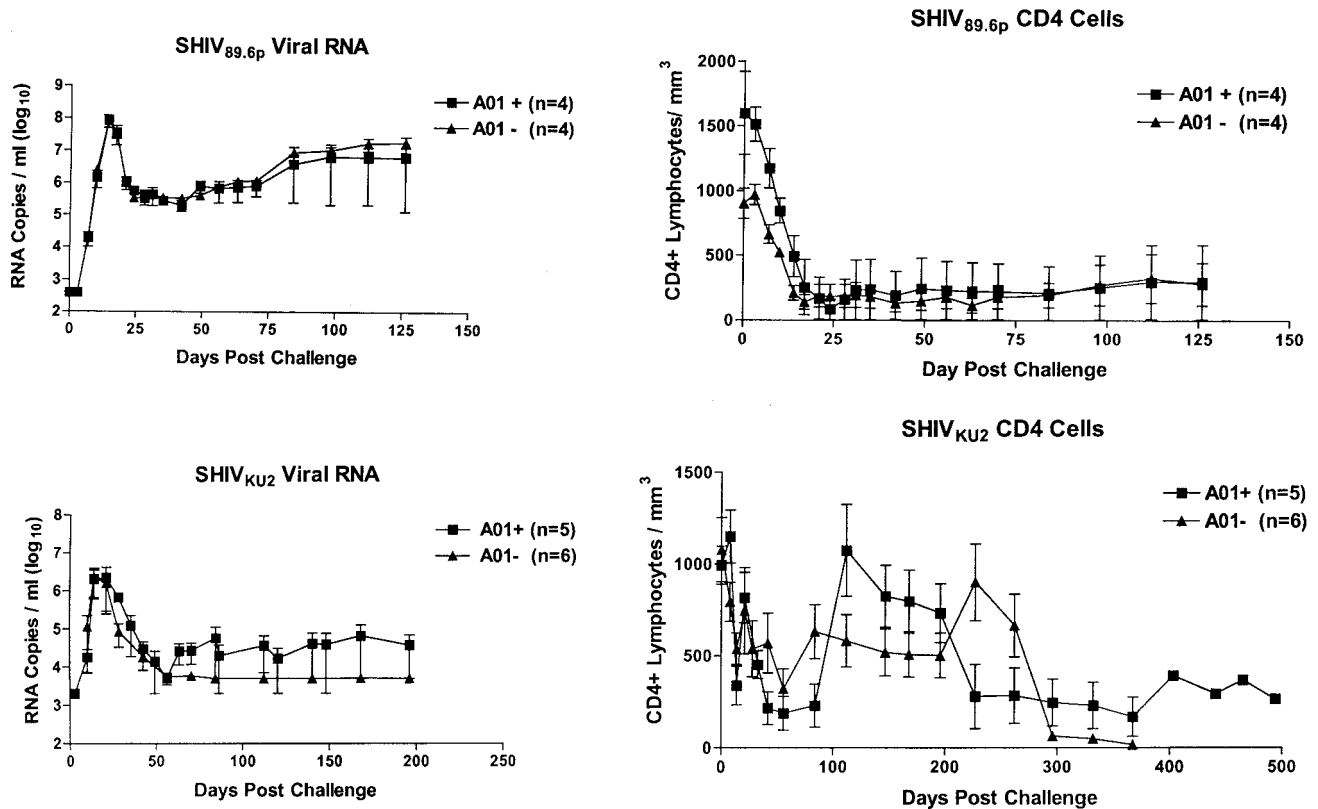


FIG. 7. Virus load and CD4<sup>+</sup> T-cell counts in macaques infected with the SHIV<sub>89.6p</sub> and SHIV<sub>KU2</sub> strains. Shown are viremia and CD4<sup>+</sup> T-cell data obtained from four Mamu-A\*01-positive and four Mamu-A\*01-negative macaques infected intravenously with SHIV<sub>89.6p</sub> (top panels) and viremia and CD4<sup>+</sup> T-cell counts obtained from five Mamu-A\*01-positive and six Mamu-A\*01-negative macaques infected intrarectally with SHIV<sub>KU2</sub> (bottom panels).

less of the route of challenge. These differences between SIV and SHIV strains are unclear but may be explained by a differential response of rhesus macaques to HIV-1 proteins (Env, Tat, Nef, Rev) present in the SHIV chimeric virus. In summary, our findings demonstrate that the ALVAC-based SIV-*gpe* vaccine protected macaques from disease induced by this highly pathogenic virus and warrant more testing of the efficacy of an ALVAC-based HIV vaccine candidate in humans.

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