wild-type recombinant vaccinia virus, viral replication in the ovaries was controlled even in the absence of anti-DIs immunity. These results suggest that the new vaccine regimen, consisting of a DNA prime and a vaccinia virus DIs boost, safely and effectively elicits anti-immunodeficiency viral immunity.

In this study, we evaluated the vaccine efficacy of the prime-boost DNA/DIs vaccine encoding the *gag/pol* gene against a challenge with a highly pathogenic SHIV using 19 macaques. We hypothesize that the efficacy is mediated not only by the effect of virus-specific cellular immunity, but also by the effect of neutralization Ab responses against the challenged virus.

Materials and Methods

Animals

Nineteen female adult cynomolgus macaques (*Macaca fascicularis*) were purchased from Japan SLC. The macaques were fed and cared for in accordance with the standard operating procedure approved by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The study was performed in the P3 facility under guidelines established by the laboratory biosafety manual of the World Health Organization (28).

Preparation of vaccine Ags and challenge virus

Plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and recombinant DIs expressing the same genes (rDIsSIV*gag/pol*) were prepared as previously described (22, 23, 29). pcDNA3.1$^-$ and rDIsLacZ were used as controls of plasmid and recombinant viral Ags, respectively. After being immunized according to the protocol, animals were challenged with SHIV-C2/1 (30–32), which was a SHIV-89.6 variant isolated at the peak of initial plasma viremia from an infected cynomolgus macaque (31). The original SHIV strain was provided by Dr. Y. Lu (Harvard AIDS Institute, Cambridge, MA) (33, 34).

Enumeration of T PBL

Fifty microliters of whole heparinized blood samples were stained with anti-human CD3 (clone HIT3a; BD Pharmingen), anti-human CD4 (clone SK3; BD Biosciences), and anti-human CD8 (clone SK1; BD Biosciences) for 15 min at 4°C. Blood samples were treated with FACS lysing solution for 15 min at 4°C, and then 50 μl of Flow Count (Beckman Coulter) was added. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 5000 CD3-positive, lymphocyte-gated events.

Intracellular IFN-γ cytokine staining

Approximately 10^6 of fresh PBMC were incubated with 0.2 μM pooled SIV Gag peptides spanning the full length of the Gag protein (AIDS Research and Reference Program, National Institutes of Health) together with 1 μg of anti-human CD28 (clone KOLT-2; Nichirei) and 1 μg of anti-human CD49d (clone 9F10; BD Pharmingen) in an appropriate volume of RPMI 1640 supplemented with 10% FBS and antibiotics for 16 h at 37°C. Then brefeldin A (Sigma-Aldrich) was added at 10 μg/ml, and the cells were incubated for an additional 4 h. After incubation, the cells were washed, stained with anti-human CD3 (clone HIT3a; BD Pharmingen) and anti-human CD8 (clone SK1; BD Biosciences) or anti-human CD4 (clone SK3; BD Biosciences) for 15 min. The cells were washed and then treated sequentially with FACS-lysing solution (BD Biosciences) and permeablizing solution (BD Biosciences) for 10 min. The cells were stained with anti-human IFN-γ-FITC (clone 45.15; Immuno Tech) for 30 min and fixed with 2% paraformaldehyde solution. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 20,000 lymphocyte-gated events, which were then analyzed with CellQuest software (BD Biosciences).

Virus-specific IFN-γ ELISPOT assay

An ELISPOT assay was performed following the method developed by Mothe and Watkins of the Wisconsin University Primate Center (35). Ninety-six-well, flat-bottom plates were coated with anti-IFN-γ mAb (clone MD-1; U-CyTech-BV) and blocked with 2% BSA in PBS. Fresh PBMC were added to the plate at 2×10^5 cells/well in triplicate and then incubated with 0.2 μM pooled SIV Gag peptides (AIDS Research and Reference Program) for 16 h at 37°C. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the washed plates, which were then incubated for 1 h at 37°C. Individual spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Zeiss) after a 15-min reaction with an activator mix (U-CyTech-BV). An SFC was defined as a large black spot with a fuzzy border (34).

Abs to SIV Gag p27 and SHIV 89.6P Env

SIV Gag- and SHIV Env-specific IgG Ab end-point titers of the macaques' sera were measured by ELISA as previously described (23, 27, 30). All samples were run in triplicate at several dilutions. In brief, 96-well ELISA plates were coated with 0.3 μg of SIV p27 Gag (Advanced Biotechnologies) or 0.2 μM pooled SHIV 89.6P Env peptides (AIDS Research and Reference Program) per well. Heat-inactivated sera were serially diluted, then added to the ELISA plates. Gag- and Env-specific Abs bound to the Ags were captured with alkaline phosphatase-labeled goat anti-mouse IgG (EY Laboratories) and *p*-nitrophenyl-phosphate disodium substrate (Invitrogen Life Technologies).

The SHIV Env-specific neutralization Ab responses induced by challenge with SHIV were analyzed as previously described (28). In brief, 10 μg/ml purified macaque IgG was incubated with 100 50% tissue culture infectious doses ($TCID_{50}$) of SHIV-C2/1, then cultured in M8166 cells. The result was compared with parallel cultures to which preimmune IgG had been added. Neutralization was expressed as the percent inhibition of SIV Gag production in the culture supernatants. Anything >20% of inhibition was considered to be an efficient neutralization response.

Quantitation of plasma viral load

Quantitation of SHIV genomic RNA copies in plasma samples was performed by real-time PCR with a TaqMan assay kit (PerkinElmer Applied Biosystems) and a PRISM 7700 sequence detection system (PerkinElmer Applied Biosystems) as previously described (30). Genomic RNA extracted from plasma samples and SIVmac239 (RNA standard; 5.4×10^4 RNA copies) were subjected to RT-PCR using an SIVmac239–1224 forward, SIVmac239–1326 reverse primer pair and an FAM-SIV-1272T probe. RNA copy numbers from plasma samples were calculated from the standard curve. Data were expressed as RNA copies per milliliter of plasma.

Flow cytometric detection of various subpopulations in CD4$^+$ T cells

Approximately 10^6 fresh PBMC were stained with anti-human CD4 (clone SK3; BD Biosciences), anti-human CD29 (clone 4B4; Beckman Coulter), and anti-human CD45RA (clone 5H9; BD Pharmingen) or with anti-human CD4 (clone *ν*-TH/I; Nichirei) and anti-human CD28 (clone KOLT-2, Nichirei). A FACSCalibur (BD Biosciences) was used to acquire 10,000 lymphocyte-gated events, which were then analyzed with CellQuest software.

Statistical analysis

Data are expressed as the mean ± SD. The data analysis was conducted using the StatView program (SAS Institute), and all reported p values are two-sided. Comparisons between groups were performed using the Kruskal-Wallis H test, followed by the Student-Newman-Keuls correction. Correlations between protection and immune levels were analyzed using Spearman's rank correlation test. A value of $p < 0.05$ was considered significant.

Results

Immunization protocol

Plasmid DNA and the recombinant vaccinia DIs viruses with the inserted *gag/pol* region of SIVmac239 were constructed as previously described (23). Southern blotting confirmed that all plasmids and viruses had the expected genomic structures, whereas Gag-specific Western blots verified the in vitro expression of SIV Gag protein in rDIsSIV*gag/pol*-infected chick embryo fibroblasts (data not shown). In this study we opted to use the three-injection regimen for DNA immunization. Because we found that both the three- and five-injection DNA immunization strategies resulted in similar levels of T cell immunities (23). A total of 19 cynomolgus macaques were divided into four groups (Fig. 1). Group 1 macaques (prime-boost group of five macaques numbered M1 to M5) received three i.m. injections (2.5 mg) of each type of SIV*gag/pol* DNA at 8-wk intervals, followed by two injections of 10^8 PFU of rDIsSIV*gag/pol*. Group 2 macaques (DNA group of five macaques numbered M6, M7, and M14 through M16) received three i.m. injections of the same dose of each type of SIV*gag/pol* DNA at

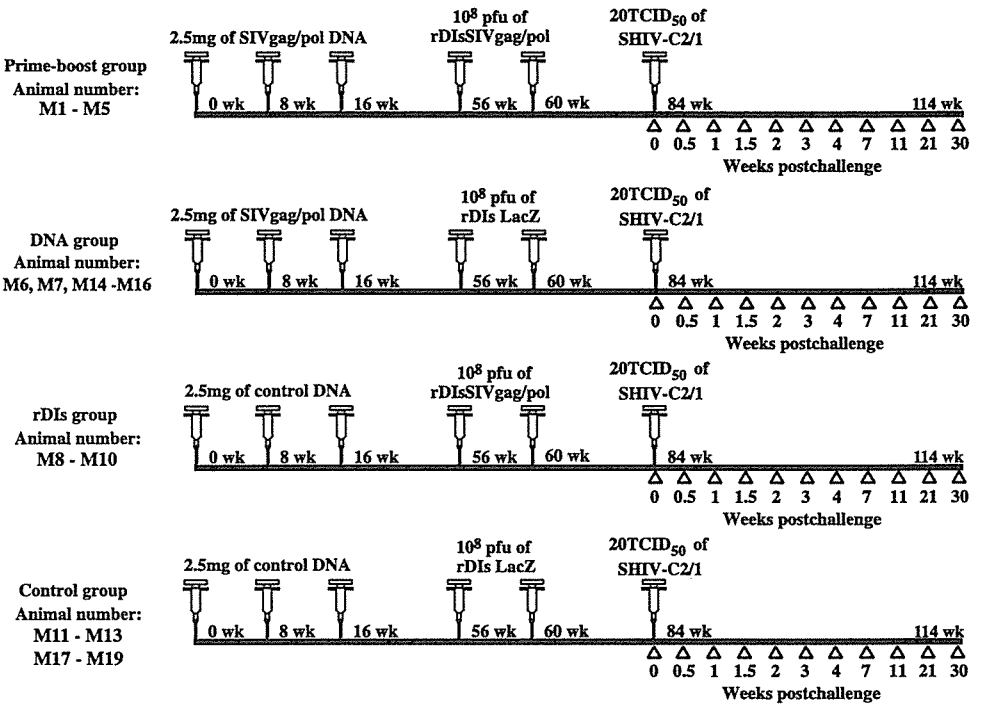

FIGURE 1. Scheme for immunization and viral challenge. Nineteen macaques were divided into four experimental groups and immunized with 2.5 mg of plasmid DNA at weeks 0, 8, and 16, then immunized with 10^8 PFU of rDIs at weeks 56 and 60. Twenty-four weeks after the final immunization, macaques were challenged with 20 $TCID_{50}$ of SHIV.

8-wk intervals, followed by two injections of 10^8 PFU of rDIsLacZ. Group 3 macaques (rDIs group of three macaques numbered M8 through M10) received three i.m. injections of control DNA pcDNA3.1$^-$ at 8-wk intervals, followed by two injections of 10^8 PFU of rDIsSIV*gag/pol*. Group 4 (control group of six macaques numbered M11 through M13 and M17 through M19) received three i.m. injections of control DNA, followed by two injections of 10^8 PFU of rDIsLacZ. Twenty-four weeks after the second booster inoculation, the macaques were i.v. challenged with 20 $TCID_{50}$ of pathogenic SHIV-C2/1, which were obtained by serum passages of SHIV-89.6. The effects of prime-boost vaccination with DNA and vaccinia DIs on protective immune induction were monitored for 30 wk, then animals were autopsied.

Induction of cellular and humoral immune responses specific for SIV Gag

We first analyzed the induction of cellular immunity by detecting the SIV Gag-specific IFN-γ ELISPOT activities of macaque PBMC after the first and third DNA primings and the first boosting of recombinant DIs in each animal (Fig. 2). A regimen of three consecutive immunizations with SIV*gag/pol* DNA induced 3- to 4-fold higher IFN-γ SFC than did a single immunization in the prime-boost and DNA groups ($p < 0.05$; Fig. 2, *A* and *B*). The numbers of IFN-γ-producing SFC increased ~8- to 9-fold after booster immunization with rDIsSIV*gag/pol* in the prime-boost group ($p < 0.01$; Fig. 2*C*). In contrast, no such increase was seen after booster immunization with rDIsLacZ in the DNA group (Fig. 2*C*). Macaques immunized with control DNA followed by rDIsSIV*gag/pol* (rDIs group) generated higher IFN-γ SFC than the DNA group ($p < 0.01$; Fig. 2, *B* and *C*). At no point in the course of immunization was Gag-specific IFN-γ SFC detected in the control group. Collectively, our findings show that the DNA/rDIs prime-boost immunization efficiently induced immunodeficiency virus-specific ELISPOT activity in macaques.

To substantiate the induction of cellular immunity specific for SIV Gag, intracellular IFN-γ staining was performed using PBMC after the first booster immunization with rDIs (Fig. 3). Of the four groups tested, the prime-boost group showed the highest frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells. The frequencies of Gag-specific IFN-γ-producing $CD8^+$ and $CD4^+$ T cell responses to Gag peptides in the prime-boost group ranged from 0.51 to 1.22% with an average of 0.82%, and from 0.37 to 0.63% with an average of 0.46%, respectively. The expression of IFN-γ-producing $CD8^+$ and $CD4^+$ T cells immunized with either SIV*gag/pol* DNA (average of $CD8^+$ T cells, 0.095%; average of $CD4^+$ T cells, 0.015%) or rDIsSIV*gag/pol* (average of $CD8^+$ T cells, 0.27%; average of $CD4^+$ T cells, 0.05%) was apparently weak (Fig. 3). Therefore, as observed for the induction of the SIV Gag-specific ELISPOT activities, the prime-boost group proved to be the most efficient of the four animal groups tested at inducing Ag-specific intracellular IFN-γ cytokine staining.

To test for the induction of humoral immunity, we assessed the SIV Gag-specific IgG titers in the animals of each group (Fig. 4). Despite the elevation of Ab titers after the first immunization with SIV*gag/pol* DNA, no enhanced responses were observed after two serial immunizations with the DNA (Fig. 4*A*). However, although the titers did not exceed 2000, enhanced Ab responses were observed after booster immunization with rDIsSIV*gag/pol*. In summary, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific cellular immune responses in immunized animals.

Enhancement of SIV-specific T cell and humoral immune responses after viral challenge

Twenty-four weeks after the second immunization with rDIs, macaques were challenged with highly pathogenic SHIV. As shown in Fig. 5*A*, Gag-specific IFN-γ SFC levels decreased on the day of challenge in all vaccinated groups, but the increase observed in the numbers of the SFC after SHIV challenge varied among the groups. The most pronounced increase was seen in the prime-boost group, with the average number of Gag-specific IFN-γ-producing cells increasing from 288/million PBMC on the day of challenge to 1124 ($p < 0.01$) 3 days after challenge. The DNA group increased from an average of 104 to 282 ($p < 0.01$), and the rDIs group from 114 to 347 ($p < 0.05$). No significant increases were noted in the control group.

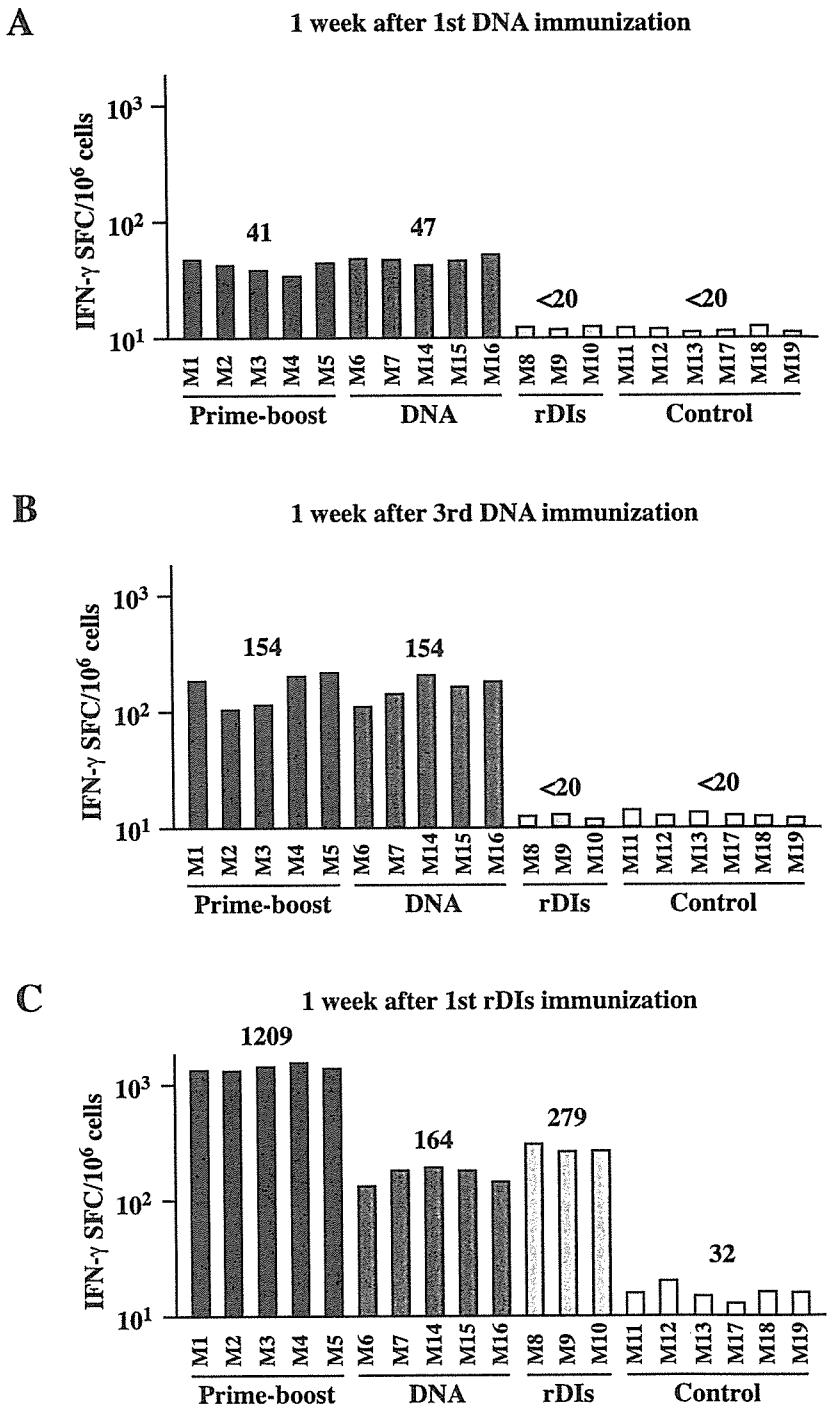

FIGURE 2. Frequency of SIV Gag-specific IFN-γ-producing cells in immunized macaques. Values are provided at 1 wk after the first DNA immunization (*A*), 1 wk after the third DNA immunization (*B*), and 1 wk after rDIs immunization (*C*). The numbers above the data bars represent the geometric means of the SFC levels in each group. Experimental groups and animal numbers are indicated at the bottom of the graph.

Intracellular IFN-γ staining of $CD8^+$ and $CD4^+$ T cells was also performed to assess any enhancement in immunodeficiency virus-specific immune responses (Fig. 5*B*). On the day of challenge, populations of Gag-specific IFN-γ-producing $CD8^+$ T cells in the prime-boost group averaged 0.32%, and populations of $CD4^+$ T cells averaged 0.11%. Three days after viral challenge, the average for Ag-specific IFN-γ-producing $CD8^+$ T cells rose to 0.61%, and that for $CD4^+$ T cells to 0.38%. Gag-specific IFN-γ-producing $CD8^+$ T cells averaged 0.18% for the DNA group and 0.25% for the rDIs group on the day of challenge, with those averages rising to 0.28 and 0.39%, respectively, by 3 days after challenge. Furthermore, the averages for Gag-specific $CD4^+$ T cells in the DNA and rDIs groups rose from 0.08 and 0.10 to 0.14 and 0.23%, respectively. The number of Ag-specific IFN-γ-producing $CD8^+$ and $CD4^+$ T cells in the control group was not affected by viral challenge. Thus, compared with the other three groups of animals, the prime-boost group showed the most significant enhancement of Ag-specific cellular immune responses after viral challenge, suggesting that Gag-specific memory T cell responses may be efficiently generated in animals by immunization with the prime-boost vaccine regimen.

To test the kinetics of humoral immune responses after SHIV challenge, we measured serum IgG titers to SIV Gag and SHIV 89.6P Env in all animals of each group (Fig. 4, *A* and *B*). The SIV Gag-specific IgG titers in all vaccinated animals were rapidly elevated and reached peak levels within 4 wk after challenge (Fig. 4*A*). The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged 14,520 ± 2,508, 5,240 ± 1,099, and 8,400 ± 1,114,

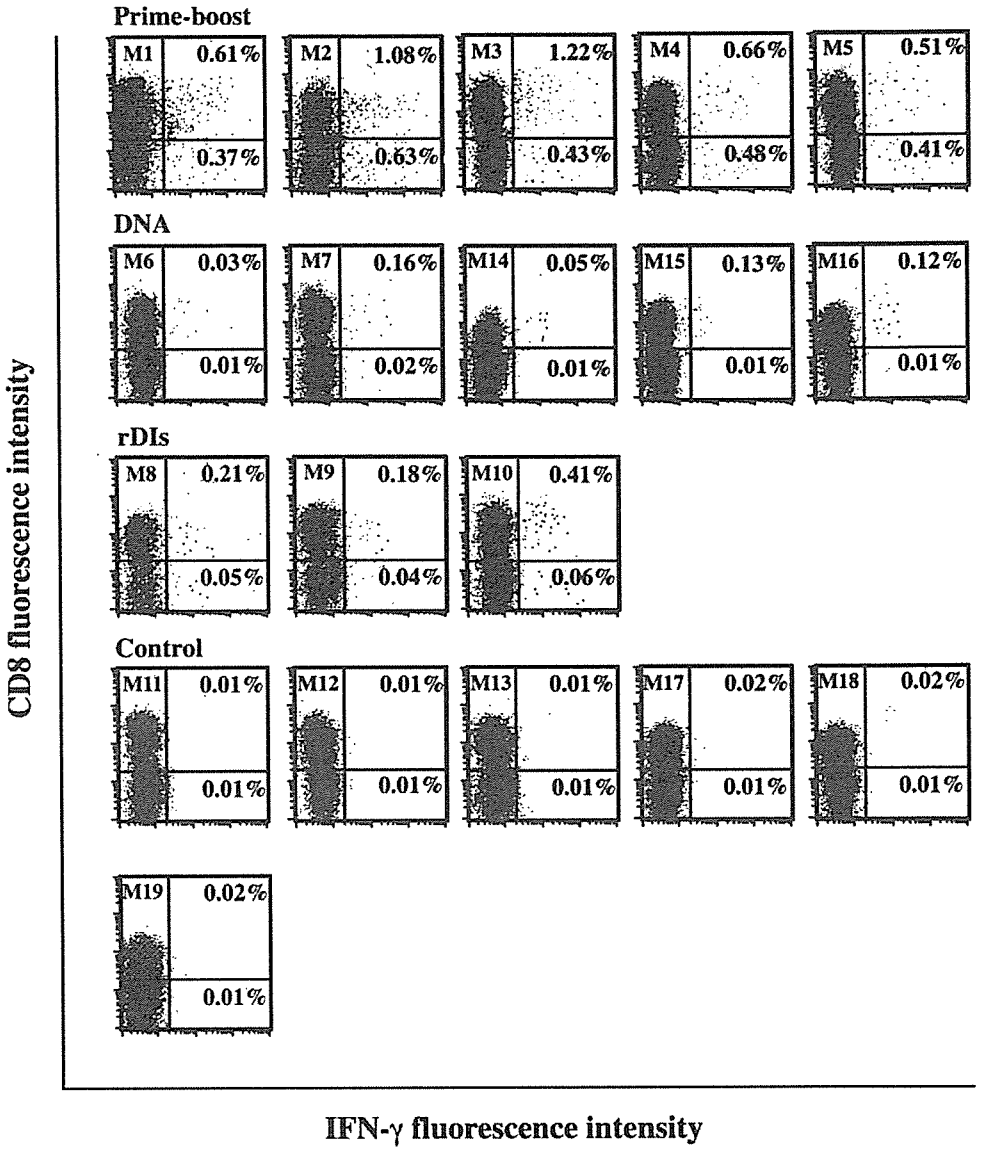

FIGURE 3. Flow cytometric analysis of SIV Gag-specific IFN-γ-producing $CD8^+$ T cells. One week after rDIs boost immunization, freshly isolated PBMC were stimulated with 0.2 μM pooled SIV Gag peptides and stained for anti-CD3, -CD8, and -IFN-γ. Twenty thousand lymphocyte-gated events were acquired. Upper and lower quadrants represent the frequencies of $CD8^+$ IFN-γ^+ and $CD4^+$ IFN-γ^+ T cells, respectively. Numbers indicate the percentage of cells in each quadrant.

respectively, with the increase in the prime-boost group reaching statistical significance ($p < 0.01$), compared with that in the DNA and rDIs groups. The Env-specific IgG appeared by 4 wk after challenge and reached peak levels between 7 and 11 wk. The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged 5,200 ± 1,839, 3,180 ± 701, and 4,533 ± 833, respectively. Both the SIV Gag- and Env-specific IgG titers in the three vaccinated groups maintained high levels and persisted throughout the challenge period. In contrast, no IgG response to Gag and Env was detected in the control group. High titers of Env-specific IgG, but only very low levels of neutralization Ab responses to SHIV-C2/1, were induced in the DNA- and rDI-vaccinated groups (Fig. 4*C*). In contrast, the prime-boost macaques, especially M1, had high levels of neutralization Ab responses (viral neutralization >70%). Thus, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific humoral responses in immunized animals and generates SHIV Env-specific binding and neutralization Abs after challenge with SHIV.

Macaques of the prime-boost group control plasma viral load and block $CD4^+$-T cell depletion

As noted above, the five macaques in the prime-boost group developed Ag-specific positive immunity after viral challenge. In these macaques, plasma viral loads were most attenuated and $CD4^+$ T cell counts best maintained in peripheral blood (Fig. 6). Peak viral loads occurred 2 wk after challenge in each group. The geometric means of the viral RNA copies were 1.1×10^7 copies in the prime-boost group, 4.7×10^7 copies in the DNA group, 4.1×10^7 copies in the rDIs group, and 4.5×10^7 copies in the control group (Fig. 6*A*). The difference observed in geometric mean peak viremia for the prime-boost and rDIs groups was significant ($p < 0.05$). Levels of peak viremia in the rDIs and control groups did not significantly differ. The peak viral loads in each had decreased by 7 wk after challenge, and the geometric means of the viral RNA copies from 7 to 30 wk were 8.1×10^3 copies (ranging from 7.1×10^3 to 9.4×10^3 copies) in the prime-boost group, 1.1×10^6 copies (ranging from 2.5×10^5 to 6.6×10^6 copies) in the DNA group, 7×10^4 copies (ranging from 5.3×10^4 to 1.1×10^5 copies) in the rDIs group, and 6.8×10^6 copies (ranging from 2.0×10^6 to 5.2×10^7 copies) in the control group (Fig. 6*A*). From 7 to 30 wk, the differences in the geometric means of the viral RNA copies between prime-boost and DNA groups ($p < 0.01$), prime-boost and rDIs groups ($p < 0.01$), and DNA and rDIs groups ($p < 0.01$) vs DNA and control groups ($p < 0.05$) were significant.

Two weeks after challenge, both DNA and control groups showed a serious depletion of $CD4^+$ T cells (to <50 cells) and a corresponding increase in viral RNA. In contrast, the prime-boost

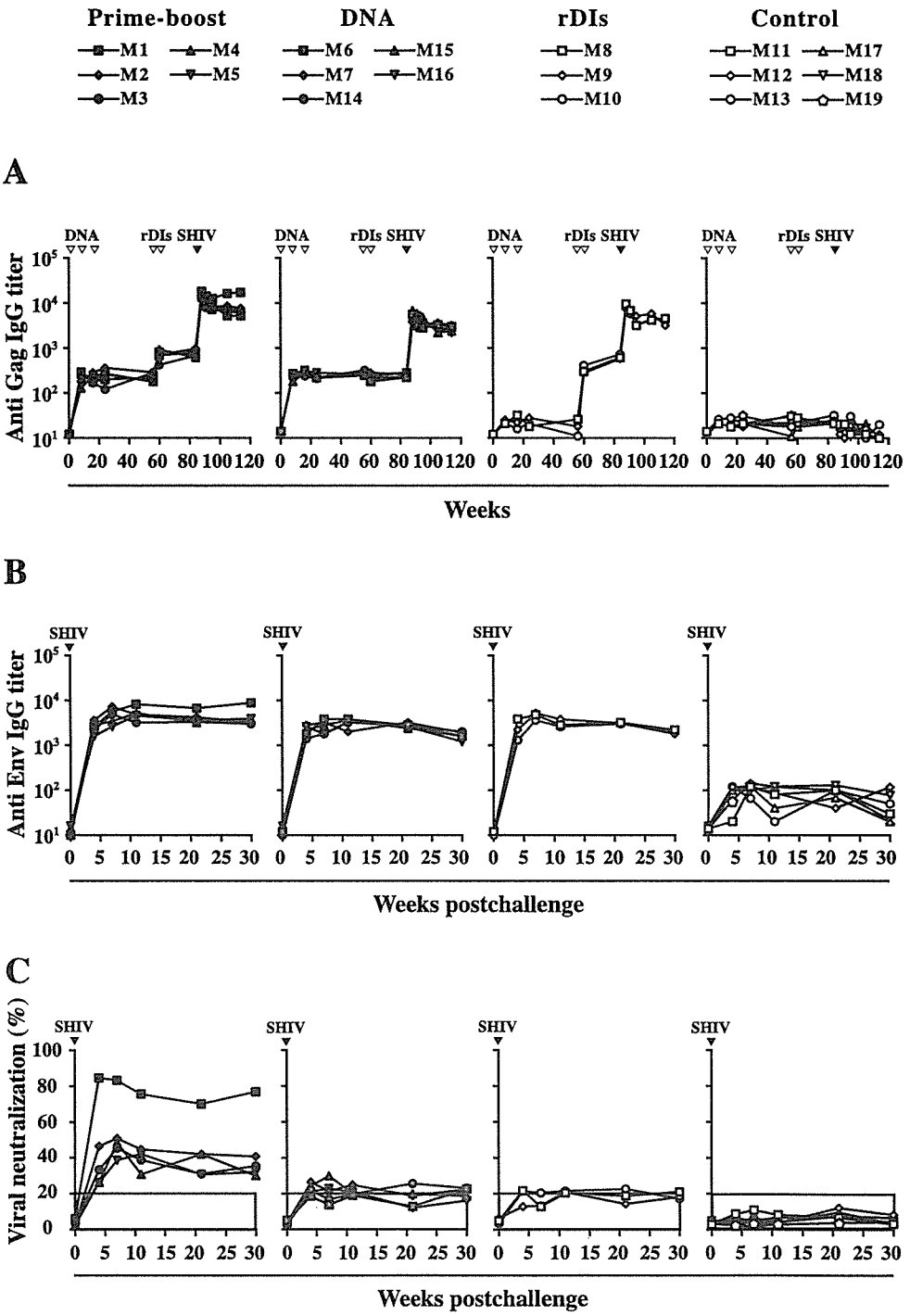

FIGURE 4. Kinetics of serum IgG titers specific to SIV Gag and SHIV 89.6P Env. *A*, SIV p27 Gag- and Env-specific IgG titers after immunization and after challenge. *B*, SHIV 89.6P Env-specific IgG titers. *C*, SHIV-specific neutralization responses. End-point titers of Gag- and Env-specific serum IgG and the percentage of SHIV-specific neutralization responses were measured at each time point. Results represent the average titer and percentage of the average viral neutralization value.

group maintained its $CD4^+$ T cell counts up to 30 wk after challenge (Fig. 6*B*). Four of the five macaques (M2–5) in the prime-boost group exhibited a gradual decrease in $CD4^+$ T cell counts; however, the macaques maintained an average of 254–303 cells from 2 to 30 wk after challenge. The one remaining macaque in the group (M1) maintained an average of 833 $CD4^+$ T cells (ranging from 630 to 1230 cells) and exhibited levels of viral RNA (<500 copies) that were undetectable except when peak viremia was reached at 2 wk (5.7×10^7 copies) and transient viral replication occurred at 7 wk (1.5×10^4 copies; Fig. 6, *A* and *B*).

To characterize the changes in the $CD4^+$ T cell subset in peripheral blood of each group after SHIV challenge, we used flow cytometric analysis to obtain an absolute count and to distinguish among the $CD29^+$, $CD45^+$, and $CD28^+$ cell subpopulations (Fig. 6, *C–E*). By 2 wk after challenge, a sharp decrease in the $CD29^+$ subset of $CD4^+$ T cells was seen in the DNA, rDIs and control groups (Fig. 6*C*). From 2 to 30 wk after challenge, the average number of this subset of cells in the DNA, rDIs, and control groups was 1.21% (ranging from 0.79 to 2.01%), 2.14% (ranging from 1.80 to 3.59%), and 1.03% (ranging from 0.55 to 1.89%), respectively. Similarly, the $CD45RA^+$ subset of $CD4^+$ T cells in the three groups rapidly declined by 2 wk after challenge, with the average of naive cells from 2 to 30 wk being 1.04% (ranging from 0.72 to 1.32%) in the DNA group, 2.83% (ranging from 1.04 to 4.78%) in the rDIs group, and 0.88% (ranging from 0.34 to 1.34%) in the control group (Fig. 6*D*). In contrast, the prime-boost group maintained the highest frequencies of both the $CD29^+$ subset, ranging from 8.0 to 9.63% with an average of 8.82% (Fig. 6*C*), and

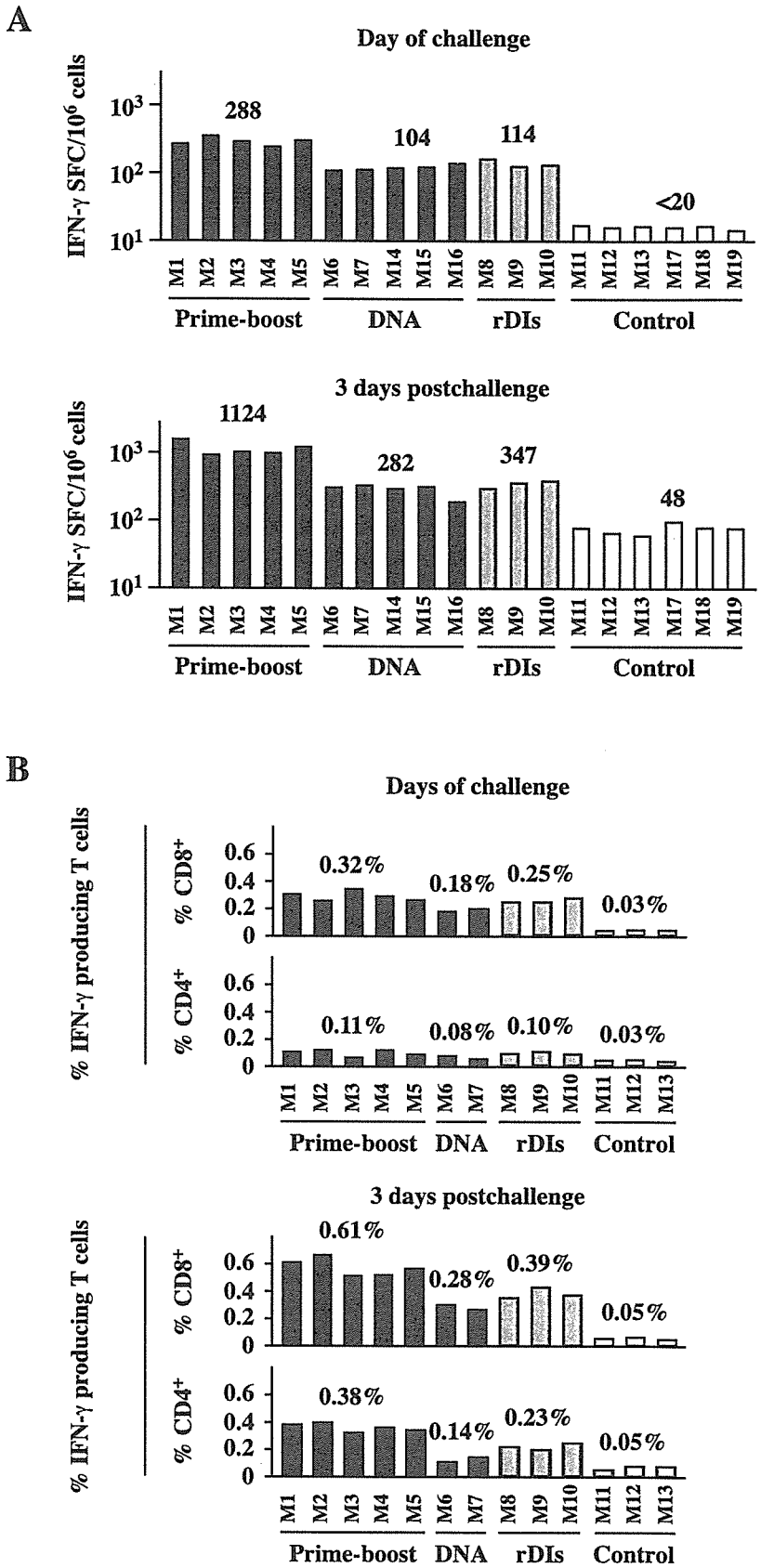

FIGURE 5. Comparison of IFN-γ ELISPOT activity and intracellular IFN-γ-producing T cells specific for SIV Gag in PBMC before and after viral challenge. *A*, ELISPOT activity. The numbers above the data bars represent the geometric means of SFC levels in each group. *B*, Intracellular IFN-γ-producing T cells. On the day of SHIV challenge and 3 days after SHIV challenge, freshly isolated cells were stimulated with SIV Gag peptides and stained for CD3, CD8, and IFN-γ. Numbers represent the percent average of the $CD4^+$ and $CD8^+$ T cell frequencies.

the $CD45^+$ subset, ranging from 6.29 to 9.16% with an average of 7.59% (Fig. 6, *C* and *D*). Flow cytometric analyses also revealed that the number of $CD4^+$ T cells expressing the costimulatory molecule CD28 rapidly dropped in the DNA, rDIs, and control groups by 2 wk after challenge (Fig. 6*E*). The average of $CD4^+CD28^+$ T cells from 2 to 30 wk after challenge in the DNA, rDIs, and control groups was 0.74% (ranging from 0.23 to 1.12%), 1.66% (ranging from 1.01 to 2.6%), and 0.91% (ranging from 0.61 to 1.13%), respectively. In contrast, $CD28^+CD4^+$ T cells in the prime-boost group ranged from 5.27 to 7.26%, with an average of

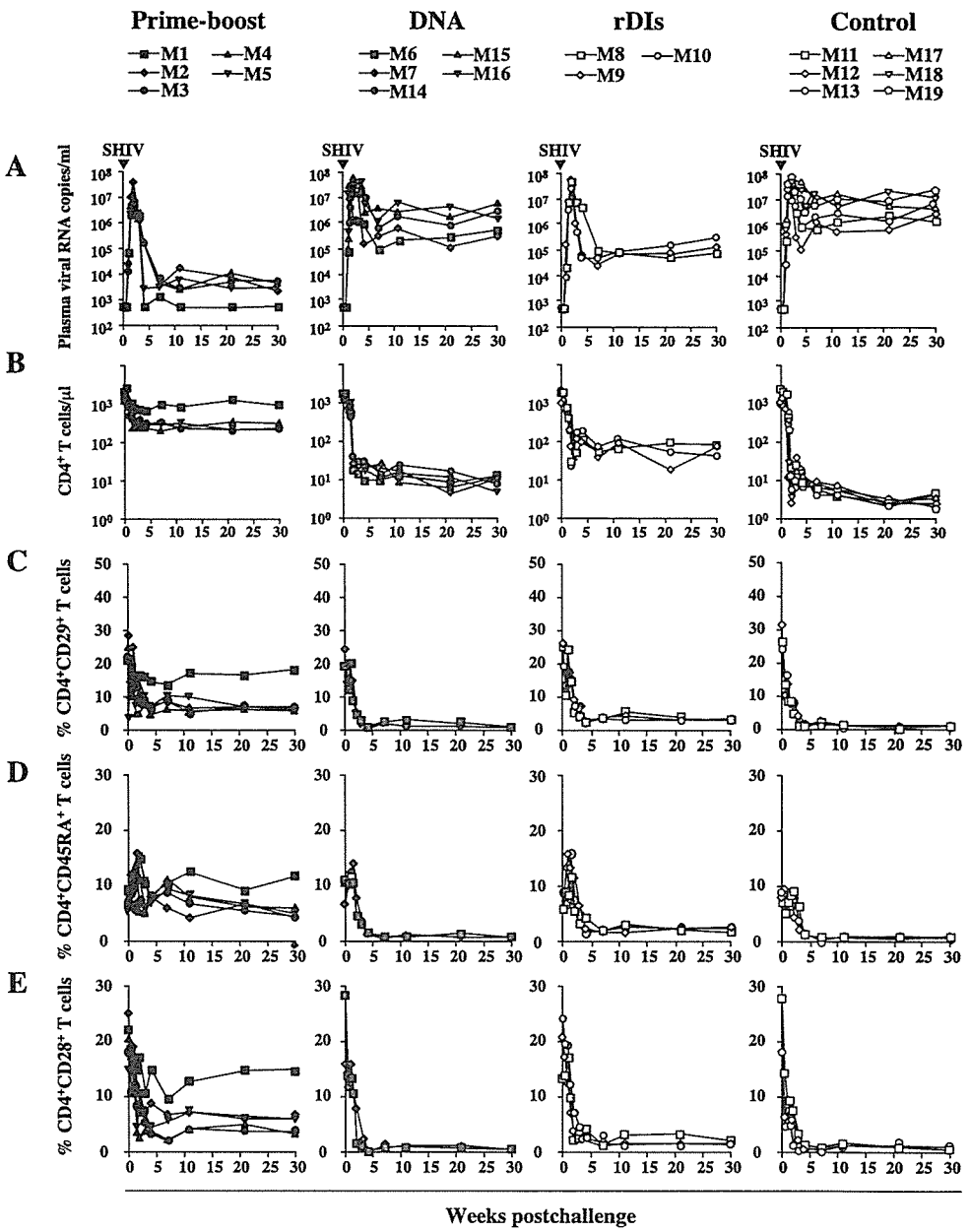

FIGURE 6. Kinetics of viral loads, $CD4^+$ T cell counts, and subpopulations of $CD4^+$ T cells in experimental groups after SHIV challenge. *A*, Plasma viral loads. Plasma viral loads were measured using the real-time PCR system. Levels <500 copies/ml were considered undetectable in this system. *B*, $CD4^+$ T cell counts. Whole blood was stained for CD3, CD4, and CD8 Abs, and $CD4^+$ T cell counts were determined using flow cytometry. *C*, $CD4^+CD29^+$ T cells. *D*, $CD4^+CD45RA^+$ T cells. *E*, $CD4^+CD28^+$ T cells. $CD4^+$ T cell subpopulations were not reduced in the prime-boost animal group.

6.75%. Thus, the prime-boost group maintained $CD29^+$, $CD45RA^+$, and $CD28^+$ cell subpopulations in $CD4^+$ T cells after viral challenge.

Controls of viremia and stability of $CD4^+$ blood lymphocytes correlate with Gag-specific IFN-γ SFC and neutralization Ab responses

Because positive immune responses were detected in the animals immunized with the prime-boost vaccine of DNA/vaccinia DIs, we examined whether any immune responses correlated with the positive immunities using Spearman's rank correlation test (Fig. 7). The set-point levels of plasma viral RNA and $CD4^+$ T cell counts 7 wk after challenge significantly correlated with the Gag-specific IFN-γ SFC levels 3 days after challenge (plasma viral RNA levels vs Gag-specific IFN-γ SFC levels: Rs = 0.850, $p = 4.07 \times 10^{-6}$; $CD4^+$ T cell counts vs Gag-specific IFN-γ SFC levels: Rs = 0.968, $p = 1.10 \times 10^{-11}$; Fig. 7*A*). Interestingly, there was less correlation between the same set-point plasma viral RNA levels and $CD4^+$ T cell counts and the neutralization Ab responses 7 wk after challenge (plasma viral RNA levels vs percent viral neutralization: Rs = 0.796, $p = 4.53 \times 10^{-5}$; $CD4^+$ T cell counts vs percent viral neutralization: Rs = 0.851, $p = 3.93 \times 10^{-6}$). No correlation at all was observed between positive immune responses and anti-Gag and anti-Env Ab titers (data not shown).

Discussion

It is believed likely that HIV-specific immune responses are associated with a decline in viral load and $CD4^+$ T cell maintenance. Our current study using the macaque model suggests that the prime-boost regimen, that is, priming with SIV*gag/pol* DNA followed by boosting with rDIsSIV*gag/pol*, modifies pathogenic SHIV infection. Furthermore, when the relationship between protection and the levels of immune responses was analyzed, we found that Gag-specific IFN-γ T cells showed a strong correlation and neutralization responses a weaker correlation with the suppression of plasma viral RNA levels and maintenance of $CD4^+$ T cell counts. These results accord with previous reports associating

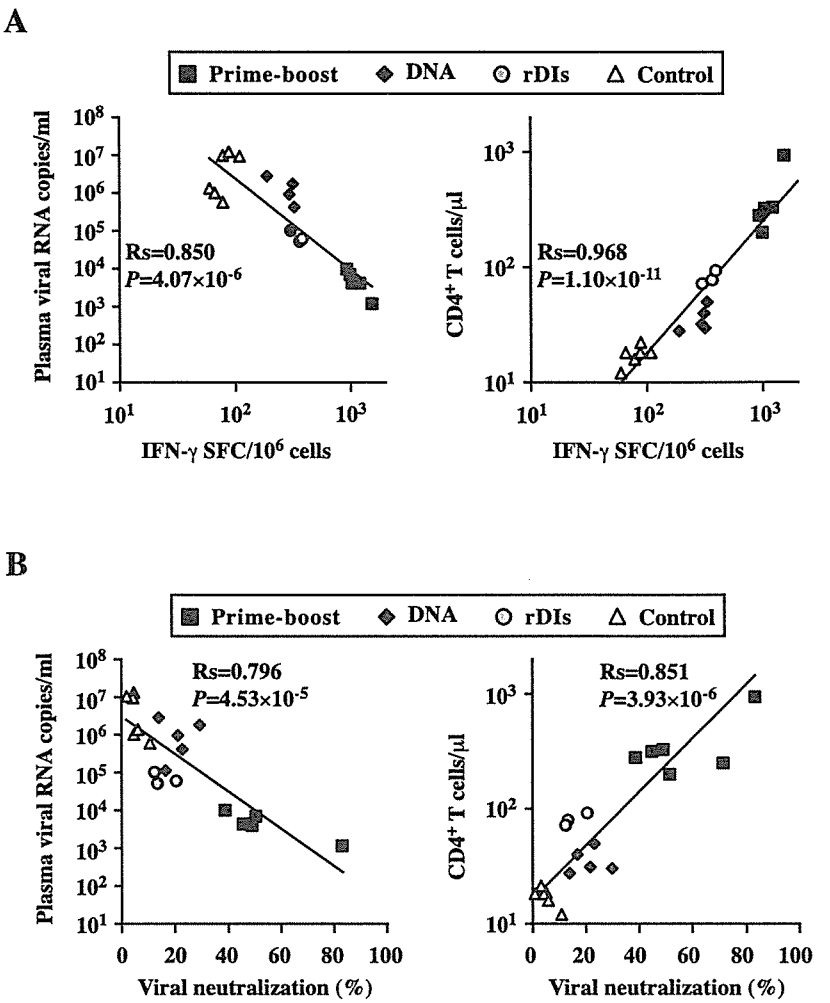

FIGURE 7. Correlations between protection and immune responses. *A*, The correlations between the decline in plasma viral RNA and the increased number of Gag-specific IFN-γ SFC, and between $CD4^+$ T cell counts and Gag-specific IFN-γ SFC. *B*, Correlations between the decline in plasma viral RNA and increased neutralization Ab responses, and between $CD4^+$ T cell counts and neutralization Ab responses. Correlations are calculated using Spearman's rank correlation test.

viral control with cellular immune responses in animals immunized with a prime-boost vaccination of either DNA/MVA (11) or cytokine-augmented DNA (36) encoding *gag* and *env* genes, followed by SHIV challenge. Neutralization Ab production was also detected in the animals (11, 36). Our new observations in vaccine research include the following: 1) Because positive immune responses better correlated with T cell than neutralization responses, it is probable that control of the plasma viral load and $CD4^+$ cell counts was achieved by virus-specific cellular immune responses. 2) Although our vaccine target was only Gag in this strategy, neutralization titers were detected in the prime-boost group that were higher than those induced in animals immunized with DNA, rDIs, or vector controls alone. These higher titers of the neutralization Ab responses against challenge virus might account for the presence of a high number of $CD4^+$ T cells in prime-boost animals (Fig. 6*B*) and might be associated with the production of neutralization Ab. It may, therefore, be reasonable to conclude that anti-Env neutralization Abs were effectively induced in the animals after SHIV challenge. Thus, we suggest that not only cellular responses, but also neutralizing Ab responses, elicited by the challenge virus may play a role in the pathogenesis of HIV/AIDS in the macaque model. 3) This vaccination regimen consisted of DNA and a nonreplicating vaccinia virus DIs, which is very safe even in immunodeficient states. Although other highly attenuated vaccinia strains replicate under synchronized viral infections to mammalian cells (37, 38), the DIs does not replicate in any mammalian cells tested because of natural big deletion of the genome (22, 23, 39). Thus, DIs vaccination eliminates the risk of a disseminated or progressive vaccinia viral infection in the immunocompromised, HIV-infected individual. Therefore, the DNA/DIs vaccine will be most safe in mammalians and may be suitable for therapeutic vaccine.

Recently, we demonstrated that priming with SIV*gag/pol* DNA, followed by boosting with rDIsSIV*gag/pol* generated both Th1-type $CD8^+$ and $CD4^+$ T cell responses specific for SIV Gag, resulting in the protection of immunized mice from a wild-type vaccinia virus recombinant expressing SIV Gag and Pol (23). Our previous mouse and macaque results (23, 40) (Fig. 6) showed that DNA alone was not as effective at inducing positive immunity in the macaque AIDS model as had been reported by others (11, 36). This discrepancy may depend on differences in DNA preparation, for example, whether the target HIV DNA was optimized to the human codon.

Although the exact immune mechanism responsible for protection from viral infection is not yet fully understood, both Ag-specific $CD4^+$ and $CD8^+$ T cell responses were clearly enhanced by viral infection in the prime-boost-immunized animals that exhibited a pronounced attenuation of plasma viral load. Our finding that challenge with the highly pathogenic SHIV virus enhances cellular immunity confirms the results of a recent study (41). It has been demonstrated that HIV-specific $CD8^+$ T cell responses play an important role in controlling viral replication by cytolysis and cytokine and/or anti-virus factor production (1, 2, 3, 42, 43). Others have also documented that HIV-specific $CD4^+$ T cell responses contribute to virus control or the slowing of disease progression (44–46). The critical role played by $CD4^+$ T cell responses against viral infections was also reported in studies of murine

lymphatic choriomeningitis viral infection (47) and CMV immunity in bone marrow transplant recipients (48). DNA/poxvirus prime-boost vaccination induced a high frequency and a high avidity of $CD8^+$ cytotoxic T lymphocyte populations (49), with the magnitude of HIV/SIV-specific $CD4^+$ and/or $CD8^+$ T lymphocyte responses in the course of infection inversely correlating with the viral load (50, 51). In addition, MHC class I molecules loading CTL epitopes may help control viral replication (52–56). The exact mechanisms underlying protective immune responses against HIV-1 remain a subject of debate; however, the above studies suggest that the simultaneous induction after vaccination of both Ag-specific $CD8^+$ and $CD4^+$ T cell responses may make it possible to attenuate immunodeficient viral infection. In this study we showed the efficacy of the prime-boost vaccination by monitoring IFN-γ ELISPOT, intracellular IFN-γ, and Ab responses. In the prime-boost group, boosting with rDIsSIV*gag/pol* induced ELISPOT responses (average of 1209 SFC) almost 10-fold higher than those induced by SIV*gag/pol* DNA (average of 154 SFC). In addition, intracellular IFN-γ staining revealed that the prime-boost vaccination generated high levels of Gag-specific intracellular IFN-γ-producing $CD8^+$ T cells (average, 0.82%; range, 0.51–1.22%) as well. However, lower Gag-specific T cell responses were observed in macaques vaccinated with either SIV*gag/pol* DNA or rDIsSIV-*gag/pol* alone than with the prime-boost regimen. In contrast to the strong Gag-specific T cell responses generated by the prime-boost vaccination, humoral responses specific for the same Ag were apparently low throughout the course of immunization. Although the peak IgG titers in the prime-boost group were observed after the first or second boosting with rDIsSIV*gag/pol*, Ab titers remained low. These results are in line with our previous study using the mouse model (23), suggesting that our prime-boost vaccine immunodominantly generates SIV Gag-specific cellular responses in macaques.

Monitoring ELISPOT and intracellular IFN-γ T cell responses specific for Gag revealed that responses decreased at the time of challenge with pathogenic SHIV, but then rapidly recovered. Gag-specific IFN-γ ELISPOT responses in the prime-boost group averaged 288 SFC on the day of challenge and increased to 1124 SFC on day 3 after challenge. The population of intracellular IFN-γ-producing $CD8^+$ and $CD4^+$ T cells specific for Gag also increased from an average of 0.32 to 0.61% and from an average of 0.11 to 0.38%, respectively, suggesting that our prime-boost vaccine generated a high frequency of very responsive $CD4^+$ and $CD8^+$ memory T cells that immediately reactivated sufficient levels of the Ag-specific immune responses against the SHIV Ag. Furthermore, a kinetic study of plasma viral loads and counts of $CD4^+$ T cells after challenge with SHIV revealed different patterns for each group. Although peak plasma viral loads were observed 2 wk after challenge in all groups, the number of plasma RNA copies peaking at that time in the prime-boost group were ~5-fold lower than in other groups, with numbers remaining depressed during the period extending from 7 to 30 wk after infection. However, high $CD4^+$ T cell counts were maintained in the prime-boost group. These results suggest a correlation between both plasma viral loads and the maintenance of high $CD4^+$ T cell counts and T cell response levels.

With regard to safety of vaccinia DIs as a vaccine vector, its viral replication occurs only in chick embryo fibroblasts, not in any mammalian cell lines tested (22, 24–26, 57). Because a vaccine regimen combining DNA and a defective DIs vector would not run the risk that the virus used as vector might replicate and disseminate, it would pose less of a risk to a severely immunocompromised host. Furthermore, in this study using the macaque model, we demonstrated that the cellular immune responses generated by the prime-boost vaccination were higher than those induced by vaccination with either DNA or rDIs alone and that response levels correlated to plasma viral loads and $CD4^+$ T cell counts after challenge with pathogenic SHIV. In summary, these results demonstrate that the new prime-boost regimen safely and effectively elicits anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1 infection as well as against HIV-induced disease progression.

Current macaque models of HIV, SIV, and SHIV may fall short of precisely mirroring human HIV infection. In some macaque HIV/AIDS models, SIVmac239 has been targeted as a desirable challenge virus, because it is a typical CCR5-tropic SIVmac and can cause both chronic and progressive disease in macaques (41, 58, 59). However, the virus is very difficult to neutralize and also very difficult to clear even from animals that have been previously immunized with homologous recombinant vector-based vaccines (41, 58, 59). Only live attenuated SIV has been reported to control SIVmac239 (T. Allen, Global HIV Vaccine Enterprise Meeting, Washington, October 21, 2004). Although there may be no macaque model suitable for evaluating the efficacy of an SIV or HIV experimental immunogen, in this study we clearly showed that vaccination with an SIV experimental immunogen consisting of SIV*gag/pol* DNA and replication-defective rDIsSIV*gag/pol* caused a pronounced attenuation of the infection caused by a highly pathogenic variant of SHIV-C2/1 in all five macaques tested. SHIV-C2/1, used as challenge virus, is a variant of SHIV 89.6. Because SHIV89.6 does not induce both a marked decline in $CD4^+$ cells and a high level of plasma viral load in cynomolgus macaques, we passaged serum from virus-infected cynomolgus macaques. The variant was obtained by the serum passages using cynomolgus macaques inoculated with SHIV89.6, and it induced high levels of viremia ($1–10 \times 10^7$ viral RNA copies/ml) and marked $CD4^+$ T cell depletion (<10 cells/μl) within 2 and 3 wk after viral inoculation (30, 31, 39). Furthermore, genomic study revealed 16 mutations of genomic DNA and 15 amino acid mutations in the Env region of parental virus. Thus, the cynomolgus AIDS model challenged with SHIV-C2/1 may represent primary HIV-1 infection in humans. These results should prove useful in determining how potent the new prime-boost vaccine regimen might be at eliciting anti-immunodeficiency virus immunity.

HIV-1 has been reported to preferentially infect $CD45RO^+CD4^+$ T cells in the early stages of infection, with the number of $CD45RA^+CD4^+$ T cells declining in later stages (60–62). Furthermore, the loss of this subpopulation of $CD4^+$ T cells during the early phase of immunodeficiency virus infection correlates to disease progression (63, 64), whereas the low $CD45RA^+CD4^+$ T cell levels in the late stages of infection correlate with an increased risk of death (65–67). The levels of $CD4^+$ T cells expressing the $CD28^+$ molecule have also been demonstrated to correlate with disease progression (68, 69). To confirm the effect of prime-boost immunity after SHIV challenge, we analyzed the kinetics of $CD4^+$ T cells expressing $CD29^+$, $CD45RA^+$, and $CD28^+$ molecules. We observed that the prime-boost group maintained the subpopulations of $CD4^+$ T cells throughout the course of infection, with an average of 8.82% $CD29^+$ cells, 7.59% $CD45\ RA^+$ cells, and 6.75% $CD28^+$ cells. In contrast, $CD4^+$ T cell populations in the other DNA and rDIs groups were reduced to <3%. These results suggest that immunization with the new prime-boost regimen induces protective immunity while maintaining the levels of the various $CD4^+$ T cell subpopulations.

In summary, our study has shown that the vaccine strategy that primes with DNA and then boosts with the replication-defective

vaccinia virus DIs generates both $CD4^+$ and $CD8^+$ T cell responses specific for SIV Gag, resulting in protection of the immunized macaques from pathogenic SHIV. However, it remains to be elucidated whether the *gag/pol*-encoding vaccine may elicit a protective effect against various viral challenges, such as CCR5-tropic viruses and other primary viruses. Nonetheless, this new regimen's twin merits of safety and efficacy position it as a promising vaccine candidate against HIV-1 infection as well as against HIV-induced disease progression.

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Disclosures

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Vaccine-based, long-term, stable control of simian/human immunodeficiency virus 89.6PD replication in rhesus macaques

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The X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P (or 89.6PD) causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system, whereas pathogenic R5-tropic simian immunodeficiency virus (SIV) infection, like HIV-1 infection in humans, results in chronic disease progression in macaques. Recent pre-clinical vaccine trials inducing cytotoxic T lymphocyte (CTL) responses have succeeded in controlling replication of the former but shown difficulty in control of the latter. Analysis of the immune responses involved in consistent control of SHIV would contribute to elucidation of the mechanism for consistent control of SIV replication. This study followed up rhesus macaques that showed vaccine-based control of primary SHIV89.6PD replication and found that all of these controllers maintained viraemia control for more than 2 years. SHIV89.6PD control was observed in vaccinees of diverse major histocompatibility complex (MHC) haplotypes and was maintained without rapid selection of CTL escape mutations, a sign of particular CTL pressure. Despite the vaccine regimen not targeting Env, all of the SHIV controllers showed efficient elicitation of *de novo* neutralizing antibodies by 6 weeks post-challenge. These results contrast with our previous observation of particular MHC-associated control of SIV replication without involvement of neutralizing antibodies and suggest that vaccine-based control of SHIV89.6PD replication can be stably maintained in the presence of multiple functional immune effectors.

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INTRODUCTION

The well-established importance of cytotoxic T lymphocyte (CTL) responses in the control of immunodeficiency virus replication has led the way to development of prophylactic AIDS vaccine regimens that augment virus-specific CTL responses (Borrow *et al.*, 1994; Koup *et al.*, 1994; Matano *et al.*, 1998; Ogg *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999; McMichael & Hanke, 2003; Goulder & Watkins, 2004). In a model of X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P or 89.6PD infection (Reimann *et al.*, 1996; Lu *et al.*, 1998), which causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system in macaques, several pre-clinical trials of prophylactic AIDS vaccines have successfully shown that efficient CTL induction results in control of virus replication and prevention of acute AIDS progression (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002; Willey *et al.*, 2003). In contrast, most trials of such CTL-based vaccines have failed to show viraemia control in models of R5-tropic simian immunodeficiency virus (SIV) infection, which result in chronic

disease progression in macaques as in human immunodeficiency virus type 1 (HIV-1) infection in humans (Feinberg & Moore, 2002; Horton *et al.*, 2002; Casimiro *et al.*, 2005). Comparison of vaccine effects on virus replication in the acute AIDS model of X4-tropic SHIV infection with those in the chronic model of R5-tropic SIV infection could contribute to the development of an effective prophylactic AIDS vaccine for control of persistent HIV-1 replication.

We have developed a prophylactic AIDS vaccine using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector boost system and have shown its potential for efficient induction of Gag-specific CTL responses in Burmese rhesus macaques (Kano *et al.*, 2002; Matano *et al.*, 2004). In preclinical trials in an acute AIDS model, all of the macaques vaccinated with the DNA-prime/SeV-Gag vector boost system controlled SHIV89.6PD replication after challenge (Matano *et al.*, 2001; Takeda *et al.*, 2003). Furthermore, a trial of the prophylactic DNA-prime/SeV-Gag boost vaccine showed control of SIVmac239 replication leading to undetectable set-point plasma viraemia in five out of eight vaccinees (referred to as SIV controllers), despite failure of virus control in the other three vaccinees (referred to as SIV non-controllers) (Matano *et al.*, 2004). All of the SIV controllers showed rapid selection of viral CTL escape mutations, and analysis of the rhesus major histocompatibility complex (MHC) suggested that SIV control was associated with particular MHC haplotypes such as *90-120-Ia* and 'elite' CTL responses specific for the MHC-restricted epitopes (Matano *et al.*, 2004). Follow up of these SIV controllers revealed that some lost this control with accumulation of multiple viral CTL escape mutations (Kawada *et al.*, 2006).

In this study, we followed up, for more than 2 years, rhesus macaques that showed vaccine-based control of SHIV89.6PD replication (referred to as SHIV controllers). Our results showed durable and stable virus control in the SHIV controllers, contrasting with our previous observation in SIV controllers.

METHODS

Animal experiments. Ten vaccinated macaques used in our previous SHIV89.6PD challenge experiments (Matano *et al.*, 2001; Takeda *et al.*, 2003) were analysed in this study. The animal list is shown in Table 1. All were Burmese rhesus macaques (*Macaca mulatta*) and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

The immunization and challenge protocols have been described previously (Matano *et al.*, 2001; Takeda *et al.*, 2003). Of the ten macaques in the SHIV89.6PD challenge experiment, three (R00-013, R00-015 and R00-017) received a single intranasal immunization with replication-competent SeV expressing SIVmac239 Gag (SeV-Gag) (Kato *et al.*, 1996; Kano *et al.*, 2002) before challenge. Two (R99-007 and R99-011) received four immunizations with FMSIV DNA followed by a single SeV-Gag booster. The FMSIV plasmid DNA used in this DNA vaccination protocol (DNAv1) was constructed from an $SHIV_{MD14YE}$ molecular clone DNA (Shibata *et al.*, 1997a) by replacing SHIV *env* with ecotropic Friend murine leukemia virus (FMLV) *env*

Table 1. Summary of the vaccinees challenged with SHIV89.6PD

Animal	MHC I haplotype*	Vaccine protocol	Set-point virus load†	Virus load around year 2‡	Gag mutations around month 2§	Gag mutations after month 6‖
R00-013	ND	SeV-Gag	10^4–10^6	10^4–10^6 (at wk 52)	ND	ND
R00-015	*90-120-Ib*	SeV-Gag	<400	<400	None	None at wk 60
R00-017	*90-030-Ih*	SeV-Gag	<400	<400	None	None at wk 58
R99-007	ND	DNAv1/SeV-Gag	<400	<400 (at wk 28)	None	ND
R99-011	*90-010-Ie*	DNAv1/SeV-Gag	<400	<400	None	None at wk 51
R99-005	*90-010-Ie*	DNAv2/SeV-Gag	<400	<400	None	None at wk 49
R99-012	*90-030-Ih*	DNAv2/SeV-Gag	<400	<400	None	None at wk 51
R00-020	*90-122-Ie*	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52
R00-023	ND	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52
R00-024	*90-120-Ib*	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52

*MHC I haplotype was determined by reference strand-mediated conformation analysis, as described previously (Arguello *et al.*, 1998; Matano *et al.*, 2004). MHC I haplotype *90-120-Ib* is derived from breeder R90-120, *90-010-Ie* from R90-010, *90-122-Ie* from R90-122 and *90-030-Ih* from R90-030. MHC I haplotypes *90-010-Ie* and *90-122-Ie* are identical.

†Plasma viral loads [RNA copies (ml plasma)$^{-1}$] around week 20.

‡Macaque R00-013 developed AIDS and was euthanized at week 53. Macaque R99-007 was euthanized at week 29 because of the limitation of available cage numbers.

§A *gag* gene fragment was amplified from plasma RNA at week 5 or from PBMC-derived DNA at week 7 or 8 and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes. The results are shown in Table 2.

‖A *gag* gene fragment was amplified from PBMC-derived DNA and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes.

ND, Not determined.

(Matano *et al.*, 2000). Two macaques (R99-005 and R99-012) received four immunizations with both the FMSIV DNA and an FMLV receptor (mCAT1)-expression plasmid DNA (Albritton *et al.*, 1989) followed by a single SeV-Gag booster. This second DNA vaccination protocol (DNAv2) has been shown to elicit efficient CTL responses by confined mCAT1-dependent FMSIV replication (Matano *et al.*, 2000). Three macaques (R00-020, R00-023 and R00-024) received a single immunization with CMV-SHIVdEN DNA (DNAv3) followed by a single boost with an F-deleted replication-defective SeV-Gag (F^-SeV-Gag) (Li *et al.*, 2000; Takeda *et al.*, 2003). This CMV-SHIVdEN plasmid DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA and had the genes encoding SIVmac239 Gag, Pol, Vif and Vpx, SIVmac239/HIV-1_{DH12} chimeric Vpr and HIV-1_{DH12} Tat and Rev (Matano *et al.*, 2004). All ten animals were challenged intravenously with 10 $TCID_{50}$ SHIV89.6PD (Lu *et al.*, 1998) approximately 3 months after the last immunization. Four unvaccinated animals were also challenged with SHIV89.6PD and all failed to control virus replication.

Quantification of plasma viral loads. Plasma RNA was extracted using a High Pure Viral RNA kit (Roche Diagnostics). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed–Muench method, as described previously (Matano *et al.*, 2004). The lower limit of detection was approximately 4×10^2 RNA copies ml^{-1}.

Sequencing of viral and proviral genomes. Plasma RNA was extracted as described above and genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a DNeasy kit (Qiagen). A fragment corresponding to nt 458–2185 (containing the entire *gag* region) in the SHIV89.6P genome (GenBank accession no. U89134) was amplified from plasma RNA by nested RT-PCR. Alternatively, fragments corresponding to nt 458–2185, 2019–3187, 3038–4197, 4056–5213, 5079–6250, 6065–7225, 7047–8176 and 7998–9172 in the SHIV89.6P genome were amplified from proviral DNA by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, PCR products were subcloned into plasmids using a TOPO cloning system (Invitrogen) and sequenced.

Measurement of virus-specific T-cell levels by intracellular cytokine staining. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (Matano *et al.*, 2001, 2004). In brief, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with a vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SHIV-specific stimulation. The pseudotyped virus was obtained by co-transfection of COS-1 cells with a VSV-G-expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA, constructed by removing the whole FMLV *env* region from the FMSIV DNA. Alternatively, PBMCs were co-cultured with B-LCLs pulsed with peptide mixture (final concentration of each peptide, 0.5–2 μM) for peptide-specific stimulation. A panel of 117 overlapping peptides (15–17 aa in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag sequence (Sigma-Aldrich) were divided into ten pools (1–10) each consisting of 11 or 12 peptides. Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3 and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ^+ T-cell frequencies from those after SHIV-specific or peptide-specific stimulation. Specific T-cell levels of <100 cells per 10^6 PBMCs were considered negative.

Measurement of virus-specific neutralizing titres. We performed a neutralizing assay for the measurement of virus-specific neutralizing titres in plasma, as described previously (Shibata *et al.*, 1997b). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ SHIV89.6PD. In each mixture, 5 μl diluted plasma was incubated with 5 μl virus. After a 45 min incubation at room temperature, each 10 μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by ELISA for detection of SIV p27 core antigen (Beckman Coulter) to determine the 100 % neutralizing end point. The lower limit of detection was a titre of 1 : 2.

RESULTS

MHC haplotypes of the SHIV controllers

In our previous SHIV89.6PD challenge experiment (Matano *et al.*, 2001; Takeda *et al.*, 2003), three animals received a single SeV-Gag vaccination alone, whilst the remaining seven animals were immunized with a DNA-prime/SeV-Gag boost vaccine before challenge (Table 1). The seven animals vaccinated with the prime–boost vaccine (R99-007, R99-011, R99-005, R99-012, R00-020, R00-023 and R00-024) were able to control virus replication, with undetectable set-point plasma viraemia. Two (R00-015 and R00-017) of the three animals vaccinated with SeV-Gag alone were also able to control viraemia, but the remaining one (R00-013) failed to control virus replication and showed acute $CD4^+$ T-cell depletion. This animal R00-013 developed AIDS and was euthanized at week 53.

In the present study, we determined the MHC class I (MHC I) haplotypes of the SHIV controllers and their viral genome sequences at around 1 or 2 months after challenge to examine whether SHIV controllers showed rapid selection of CTL escape mutations as observed in our previous analysis, in particular MHC-associated control of SIV replication. Importantly, control of SHIV89.6PD replication was observed in vaccinees with diverse MHC haplotypes (Table 1). Analysis of the proviral *gag* region in PBMCs at around week 8 showed a predominance of the wild-type sequence in all nine SHIV controllers (Table 2). Sequencing of the plasma viral *gag* region at week 5 in three of them confirmed the lack of dominant mutations (Table 2). Thus, the SHIV controllers controlled virus replication without rapid selection of CTL escape mutations.

Follow-up of the SHIV controllers

We followed up eight of the nine SHIV controllers except for one animal, R99-007, which was euthanized at week 29 because of a limitation on available cage numbers (Table 1). All eight SHIV controllers maintained control of virus replication for more than 2 years (Fig. 1). Viraemia was undetectable and peripheral $CD4^+$ T-cell counts were maintained during the observation period. Analysis of the *gag* region in PBMC-derived proviral DNA revealed that the wild-type sequence was still dominant around 1 year after challenge in all eight (Table 1). Additionally, we succeeded

Table 2. Mutations in SHIV *gag* at 1 or 2 months post-challenge

A *gag* gene fragment was amplified by nested PCR from PBMC-derived DNA at week 7 (in R00-017) or week 8 (in others) or by nested RT-PCR from plasma RNA at week 5. The viral *gag* fragment was amplified from plasma RNA in only three of the nine SHIV controllers (R00-017, R00-023 and R00-024); this was due to lower viral loads at week 5 in the remaining SHIV controllers.

Animal	Frequency*	Position of Gag changes (aa)†
Proviral DNA at week 7 or 8		
−R00-015	8/9	None
	1/9	373
−R00-017	9/10	None
	1/10	384
−R99-007	8/9	None
	1/9	485
−R99-011	9/10	None
	1/10	141
−R99-005	8/9	None
	1/9	495
−R99-012	8/10	None
	1/10	210
	1/10	372, 456
−R00-020	9/9	None
−R00-023	7/10	None
	3/10	385
−R00-024	7/7	None
Plasma RNA at week 5		
−R00-017	2/9	None
	1/9	49
	1/9	208
	1/9	443
	1/9	49, 103
	1/9	270, 448
	1/9	59, 232, 293
	1/9	391, 481, 499
−R00-023	2/11	None
	2/11	218
	1/11	27
	1/11	434
	1/11	444, 493
	1/11	76, 182, 379
	1/11	118, 272, 380
	1/11	5, 140, 312, 434
	1/11	6, 17, 112, 205
−R00-024	2/9	None
	1/9	227
	1/9	42, 301
	1/9	272, 434
	1/9	9, 48, 367
	1/9	50, 176, 247
	1/9	103, 364, 386
	1/9	108, 137, 364, 386, 411

*Number of clones with change(s)/total number of clones.
†Amplified *gag* fragments were subcloned into plasmids for sequencing and the positions of amino acid changes in SHIV Gag in each clone are shown.

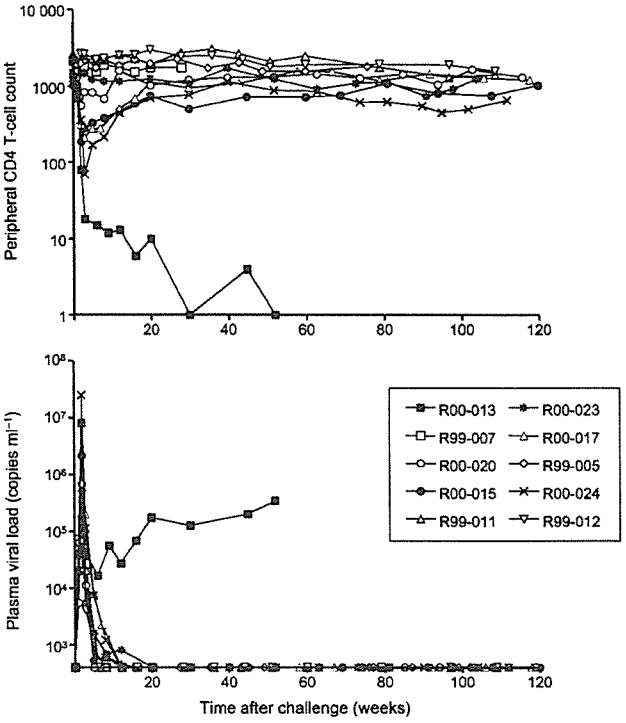

Fig. 1. Follow-up of vaccinated macaques after SHIV89.6PD challenge. Macaque R00-013 was a non-controller that failed to control virus replication, with acute $CD4^+$ T-cell depletion, whereas the other nine animals were SHIV controllers. (a) Peripheral $CD4^+$ T-cell counts μl^{-1}. (b) Plasma viral loads [viral RNA copies (ml plasma)$^{-1}$].

in amplifying almost the entire coding region of the proviral genomes from three (R00-015, R00-017 and R00-023) of the eight controllers at around 1 year for sequencing and found no dominant non-synonymous mutations except for one leading to a change in aa 401 in Env in macaque R00-015, suggesting inefficient virus replication during the period of SHIV control.

Virus-specific T-cell responses

We next examined changes in virus-specific T-cell frequencies during the period of SHIV89.6PD control. The SHIV controllers did not rapidly lose SHIV-specific T cells but most showed a gradual decrease in SHIV-specific T-cell levels, except for macaque R99-011, which maintained constant SHIV-specific $CD8^+$ T-cell levels (Fig. 2). Thus, none of the SHIV controllers showed a significant increase in SHIV-specific T-cell levels, suggesting stable virus control without any sign of a virus replication burst in the chronic phase.

In addition to virus-specific T-cell levels, we examined epitopes that were recognized by CTLs. We focused on two SHIV controllers, R00-015 and R00-017, that were vaccinated with SeV-Gag alone and examined CTL responses

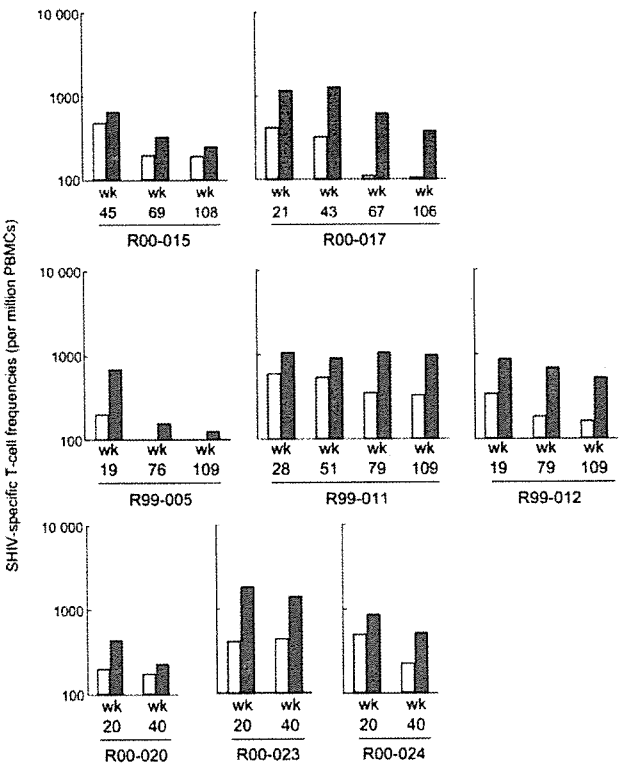

Fig. 2. SHIV-specific T-cell levels in SHIV controllers at various time points after challenge. The frequencies of SHIV-specific $CD4^+$ T cells (open bars) and $CD8^+$ T cells (shaded bars) in PBMCs are shown.

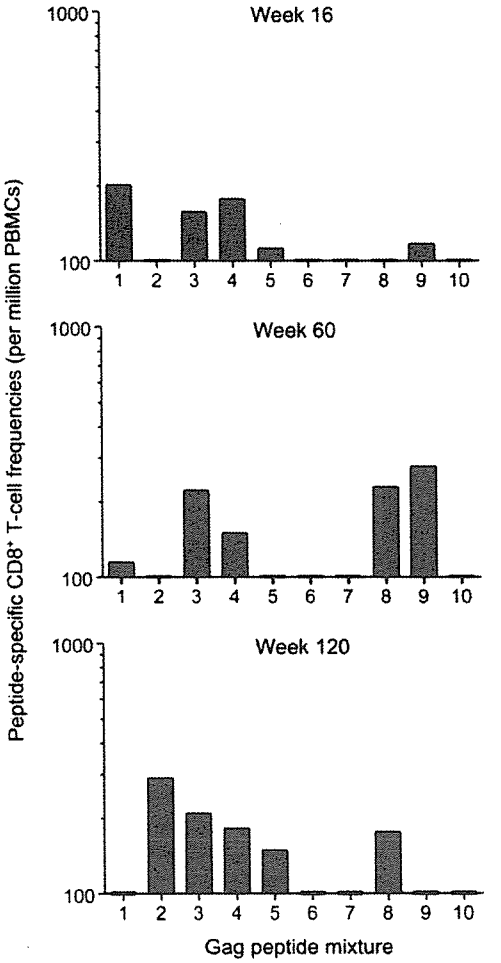

Fig. 3. Changes in frequencies of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-015 during the period of virus control. The frequencies at week 16 (top panel), week 60 (middle panel) and week 120 (bottom panel) after SHIV89.6PD challenge are shown. A panel of overlapping peptides spanning the entire SIV Gag sequence was divided into ten pools: 1 (aa 1–65), 2 (aa 55–114), 3 (aa 104–165), 4 (aa 155–213), 5 (aa 202–265), 6 (aa 255–316), 7 (aa 306–364), 8 (aa 354–416), 9 (aa 406–464) and 10 (aa 453–510). Each pool was used for stimulation to detect peptide-pool-specific $CD8^+$ T cells.

specific for ten pools of Gag-overlapping peptides. In macaque R00-015, significant Gag peptide pool 1-specific $CD8^+$ T-cell responses were detected at week 16 but became undetectable by week 120, whereas pool 8- and 2-specific $CD8^+$ T-cell responses that were undetectable at week 16 appeared at week 60 or 120, respectively, and pool 3- and 4-specific $CD8^+$ T-cell responses were detectable throughout the observation period (Fig. 3). A similar pattern of disappearance (pool 10-specific), appearance (pool 3- and 9-specific) and maintenance (pool 6- and 8-specific) of $CD8^+$ T-cell responses during the period of SHIV control was also observed in macaque R00-017 (Fig. 4). These results suggested that SHIV89.6PD replication was not completely contained in these macaques.

Virus-specific neutralizing antibody responses

We next examined virus-specific neutralizing antibody responses by determining the end-point plasma titres required to neutralize the replication of 10 $TCID_{50}$ of virus on MT4 cells. Our vaccine regimens did not utilize Env as an immunogen and no neutralizing antibody responses were induced before challenge in any of the vaccinees, as expected. Remarkably, however, SHIV89.6PD-specific neutralizing antibodies appeared rapidly between weeks 3 and 6 post-challenge and were maintained during the observation period in all of the SHIV controllers (Fig. 5). In contrast to such efficient induction of neutralizing antibodies in SHIV controllers, macaque R00-013, which failed to control SHIV replication, showed no neutralizing antibody induction after challenge.

DISCUSSION

Long-term control of X4-tropic pathogenic SHIV has been reported in follow-up studies of several pre-clinical AIDS

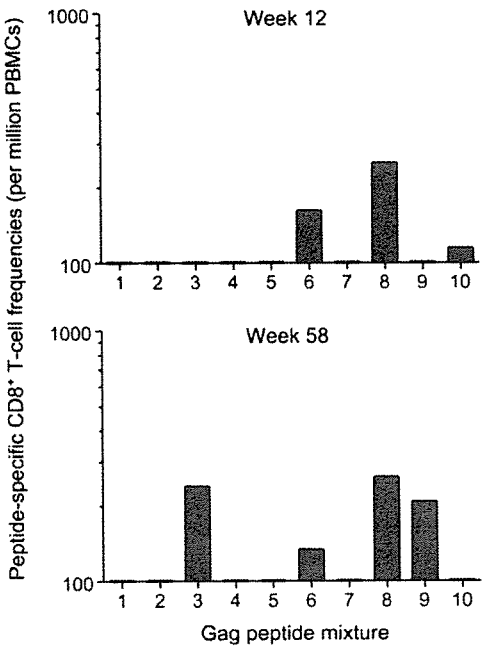

Fig. 4. Changes in frequency of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-017 during the period of virus control. The frequencies at week 12 (top panel) and week 58 (bottom panel) after SHIV89.6PD challenge are shown. Ten pools of Gag peptides were used for stimulation to detect peptide pool-specific $CD8^+$ T cells, as described in the legend to Fig. 3.

vaccine trials (Willey *et al.*, 2003; Sadagopal *et al.*, 2005). Whilst these vaccine regimens utilized Env as an immunogen (Amara *et al.*, 2002), we have developed vaccine regimens not targeting Env and demonstrated their efficacies leading to control of SHIV89.6PD replication in rhesus macaques (Matano *et al.*, 2001; Takeda *et al.*, 2003). In the present study, we followed up these SHIV controllers for more than 2 years after challenge. All maintained this control with undetectable plasma viraemia, indicating that efficient CTL induction by a prophylactic AIDS vaccine not targeting Env can result in sustained control of virus replication and protection from AIDS progression in a model of X4-tropic SHIV infection.

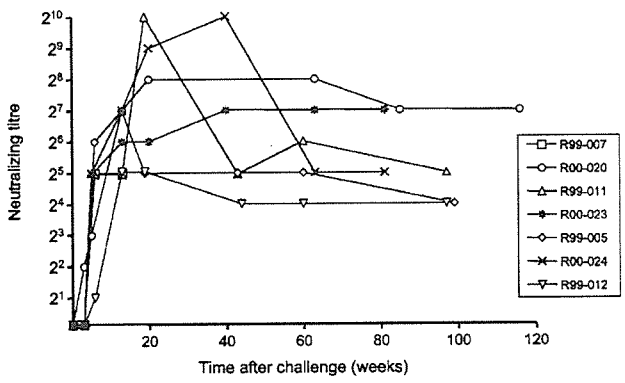

Fig. 5. SHIV89.6PD-specific neutralizing antibody levels in plasma of SHIV controllers. Plasma titres that neutralized replication of 10 $TCID_{50}$ SHIV89.6PD in seven out of nine SHIV controllers are shown. In the remaining SHIV controllers, R00-015 and R00-017, we confirmed induction of neutralizing antibodies at weeks 5, 12 and 20, but their titres were not determined.

X4-tropic SHIV and R5-tropic SIV target different $CD4^+$ T-cell subsets in rhesus macaques and this difference has been indicated as resulting in their divergent clinical courses (Nishimura *et al.*, 2004). Indeed, it has been shown that X4-tropic SHIV targets $CXCR4^+$ naive $CD4^+$ T cells for depletion, whereas R5-tropic SIV, like HIV-1 infection in humans, eliminates $CCR5^+$ effector memory $CD4^+$ T cells in rhesus macaques during the acute phase of infection (Picker *et al.*, 2004; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Nishimura *et al.*, 2005; Picker & Watkins, 2005). In the latter chronic AIDS model, several CTL vaccine trials have recently shown partial reductions in viral loads with amelioration of acute memory $CD4^+$ T-cell loss, but this partial control was transient and unstable (Letvin *et al.*, 2006; Mattapallil *et al.*, 2006; Wilson *et al.*, 2006). In our previous study (Matano *et al.*, 2004), SIV control was observed consistently in the three vaccinees possessing MHC I haplotype *90-120-Ia*, but this control was not stable and two of them lost viraemia control around week 60 after challenge. In the present study showing long-term, stable SHIV control, we found several differences between X4-tropic SHIV controllers and R5-tropic SIV controllers.

First, patterns of *de novo* neutralizing antibody induction were completely different between the two. Although the vaccine regimens did not target Env, SHIV-specific neutralizing antibodies appeared rapidly and became detectable by week 6 post-challenge in the SHIV controllers, whereas no neutralizing antibody induction was observed in the SHIV non-controllers. Thus, SHIV-specific neutralizing antibodies can be rapidly induced if animals are protected by CTLs from complete $CD4^+$ T-cell depletion in the acute phase and may be involved in viraemia control at the set point and after (Rasmussen *et al.*, 2002). In contrast, SIV-specific neutralizing antibody induction in the SIV controllers was poor and less efficient than the SIV non-controllers (data not shown), indicating that neutralizing antibody responses are not involved in SIV control.

Secondly, all of the SIV controllers showed rapid selection of viral CTL escape mutations, whereas this sign of particular CTL pressure (Borrow *et al.*, 1997; Goulder *et al.*, 1997; Price *et al.*, 1997; Goulder & Watkins, 2004; Matano *et al.*, 2004) was not observed in any of the SHIV controllers. Additionally, SIV control was associated with some MHC haplotypes such as *90-120-Ia*, but SHIV control was observed in vaccinees with diverse MHC haplotypes. Indeed, none of the SHIV controllers had the MHC

haplotype *90-120-Ia* associated with SIV control. Although the involvement of functional virus-specific $CD4^+$ T-cell responses remains unclear, these results support the notion that multiple target-specific CTL effectors are involved in SHIV control, whereas relatively limited regions of viral antigens are targeted by effectors responsible for SIV control.

All of the SHIV controllers maintained virus control for more than 2 years. Sequencing of viral genomes revealed a predominance of the wild-type sequence around 1 year after SHIV89.6PD challenge, and analysis of SHIV-specific T-cell levels showed no signs of a burst of virus replication during the chronic phase. These results indicated stable virus control in the chronic phase in the SHIV controllers. Interestingly, however, analysis of Gag peptide-specific $CD8^+$ T-cell responses in some of the SHIV controllers showed a shift of targeting epitopes during the period of virus control, suggesting that virus replication was inefficient but not completely contained, even in the SHIV controllers.

In summary, the present study revealed several differences in vaccine-based virus control in a model of X4-tropic SHIV compared with R5-tropic SIV infections. Our results suggest that, compared with virus control with limited effectors in SIV controllers, the control of X4-tropic SHIV89.6PD replication may be maintained more stably in the presence of multiple functional immune effectors.

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Effects of Immunization with CCR5-Based Cycloimmunogen on Simian/HIV$_{SF162P3}$ Challenge[1]

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A synthetic cycloimmunogen targeting the HIV-1 coreceptor CCR5 was evaluated for its capacity to induce CCR5-specific Abs with anti-HIV-1 activity in cynomolgus macaques. The cyclic closed-chain dodecapeptide (cDDR5) mimicking the conformation-specific domain of human CCR5 was chemically prepared, in which the Gly-Glu dipeptide links the amino and carboxy termini of the decapeptidyl linear chain (Arg^{168} to Thr^{177}) derived from the undecapeptidyl arch (Arg^{168} to Cys^{178}) of extracellular loop-2 in CCR5. The immunization of cynomolgus macaques with the cDDR5-conjugated multiple-Ag peptide (cDDR5-MAP) induced anti-cDDR5 serum production for ~15 wk after the third immunization. The antisera raised against cDDR5-MAP reacted with both human and macaque CCR5s, and potently suppressed infection by the R5 HIV-1 laboratory isolate (HIV$_{JRFL}$), R5 HIV-1 primary isolates (clade A:HIV$_{93RW004}$ and clade C:HIV$_{MJ4}$), and a pathogenic simian/HIV (SHIV$_{SF162P3}$) bulk isolate in vitro. To examine the prophylactic efficacy of anti-CCR5 serum Ab for acute HIV-1 infection, cynomolgus macaques were challenged with SHIV$_{SF162P3}$. The cDDR5-MAP immunization attenuated the acute phase of SHIV$_{SF162P3}$ replication. The geometric mean plasma viral load in the vaccinated macaques was 217.10 times lower than that of the control macaques at 1 wk postchallenge. Taken together, these results suggest that cDDR5-MAP immunization is an effective prophylactic vaccine strategy that suppresses and delays viral propagation during the initial HIV-1 transmission for the containment of HIV-1 replication subsequent to infection. *The Journal of Immunology,* **2006, 176: 463–471.**

Although numerous trials on preventive HIV-1 vaccines are ongoing (International AIDS Vaccine Initiative report, ⟨www.iavi.org/iavireport⟩) or being planned, a major obstacle to the development of an HIV-1 vaccine is unfortunately the marked genetic diversity of HIV-1 (1). To contend with this issue, some attempts have been made to match candidate vaccines with strains prevalent in sites in which phase III efficacy trials are to be conducted (2–4). Other strategies include the use of mixture vaccines containing Ags representative of several genetic subtypes (5, 6), the design of candidate vaccines targeting conserved HIV-1 epitopes (7, 8), and the use of candidate vaccines based on the consensus or ancestor sequences selected to minimize genetic differences between vaccine strains and current isolates (2, 9).

As alternative approaches for developing HIV-1 vaccine, other attempts have also been made to induce anti-CCR5 Abs that can bind native CCR5 and block viral infection because CCR5 is genetically stable, unlike viral targets that may rapidly mutate during the course of infection (10–14), and has been considered important in HIV-1 transmission on the basis of the findings that individuals homozygous for a 32-bp deletion in the CCR5-coding region have a very low susceptibility to HIV-1 infection (15–19). Furthermore, CCR5 is also considered as a redundant molecule in adults because CCR5-defective individuals have normal inflammatory and immune reactions (20). In fact, it is reported that CCR5-specific autoantibodies that strongly block HIV infection are induced in the sera of HIV-seronegative individuals (referred to exposed seronegative (ESN)[4] subjects) despite multiple exposures to HIV-1 (21). Therefore, CCR5 may be an important target for developing a more effective HIV-1 vaccine.

In this study, we developed a CCR5-based cycloimmunogen that can elicit an anti-CCR5 autoantibody to reconstruct the immune response induced in ESN subjects, and examined the in vivo protective effects of vaccination on acute viral infection in cynomolgus macaques, as well as the duration and magnitude of autoantibody induction.

Materials and Methods

Preparation of cyclic closed-chain dodecapeptide (cDDR5)-MAP and biotinylated cDDR5

A CCR5-derived linear dodecapeptide (linear DDR5, H_2N-ERSQKEGL HYTG-COOH), in which all side-chain groups are protected, was synthesized using an automatic peptide synthesizer and cyclized, as previously described (10). The free β-carboxyl group of Glu_1 in the protected cDDR5 was

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[4] Abbreviations used in this paper: ESN, exposed seronegative; cDDR5, cyclic closed-chain dodecapeptide; MOE, molecular operating environment; SHIV, simian/HIV; TCID, tissue culture infective dose; UPA, undecapeptidyl arch; wpim, weeks postinitial immunization.

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